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Microbial Communities and Their Impact on Bioenergy Crops in Dynamic Environments

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MICROBIAL COMMUNITIES AND THEIR IMPACT ON BIOENERGY CROPS IN DYNAMIC ENVIRONMENTS

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Biological Sciences degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this dissertation does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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LIST OF ABBREVIATIONS

AM .....Arbuscular mycorrhiza(l)

AMF .....Arbuscular mycorrhizal fungi

ANOVA .....Analysis of variance

BLAST .....Basic local alignment search tool

bp.....Base pair

C.....Celsius

DEG .....Differentially expressed gene

DNA.....Deoxyribonucleic acid

FDR.....False discovery rate

ID .....Identifier

M.....Molar

OTU .....Observational taxonomic unit

PCG.....Prairie cordgrass

PcoA.....Principal coordinates analysis

PERMANOVA .....Permutational multivariate analysis of variance

PGP .....Plant growth promotion

QC.....quality check

RI.....*Rhizophagus irregularis* DAOM 197198

RNA .....Ribonucleic acid

RNA-seq .....RNA sequencing

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ABSTRACT

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Native perennial grasses, such as prairie cordgrass (PCG, *Spartina pectinata* Link), and switchgrass (SG, *Panicum virgatum* L.) have a great potential as bioenergy crops, because they require fewer inputs, produce more energy, and reduce greenhouse gas emissions in comparison to annual cropping systems such as corn and soybean. SG has been selected by the U.S. Department of Energy for development as bioenergy crop, but studies have shown that PCG can outcompete switchgrass in terms of biomass production. These crops can also form associations with a wide variety of plant growth promoting microbes including arbuscular mycorrhial (AM) fungi.

In CHAPTER 2, we examined the beneficial microbial communities of PCG across the Upper Midwest. PCG samples were taken across Iowa, Minnesota, Nebraska, and South Dakota from a variety of environments. A high-throughput amplicon sequencing approach was used. DNA from above- and below-ground PCG tissue was extracted and amplicons targeting prokaryotic, eukaryotic, and AM fungal communities were generated. Our findings show a broad array of beneficial microbes located in PCG including well-known AM fungal species. These findings confer prior microbial surveys of PCG found in Brookings County. Alpha- and beta-diversity analysis of microbial communities indicate decreased diversity and community structure of PCG samples taken from riparian areas across the Upper Midwest.

In CHAPTER 3, we investigated the impact of AM communities on the biomass production of PCG genotypes found in the Midwest. We found high genotypic variability in the biomass potential under different nutrient supply conditions and in the mycorrhizal responsiveness of different PCG genotypes. Mycorrhizal benefits were correlated to an improved phosphate but not nitrogen nutrition of the plants.

In CHAPTER 4, we examined the impact of the AM fungus, *Rhizophagus irregularis* DAOM197198 on differential expression of mycorrhizal responsive genes in the leaves of PCG, SG, and the model grass species, *Brachypodium distachyon* under two nutrient input conditions. Our results show variations in the transcriptomes of each mycorrhizal grass species under low- and high-input nutrent conditions. Changes to carbohydrate metabolism, photosynthesis, sugar transporters, nutrient transporters, and response to disease signalling were most notably observed between these two nutrient conditions.

In CHAPTER 5, we review the current status of inter- and intraspecific diversity of AM fungi. The 450-million-year-old AM symbiosis is formed by the majority of land plants and plays a critical role for nutrient uptake, and abiotic (drought, salinity, and heavy metals) and biotic stress resistance of the host. The fungal extraradical mycelium takes up nutrients, such as phosphate and nitrogen, and delivers them to the intraradical mycelium, where the fungus exchanges these nutrients against carbon from the host. It is known for decades that AM fungi can improve the nutrient acquisition of many important crops under low input conditions, and are able to increase plant productivity in stressful environments. However, despite their application potential as biofertilizers and bioprotectors, AM fungi have so far not been widely adopted. This is mainly due to the

high variability and context-dependency of mycorrhizal growth and nutrient uptake responses that make benefits by AM fungal communities difficult to predict. In this review, we summarize our current understanding of interspecific and intraspecific fungal diversity in mycorrhizal growth benefits, and discuss the role of fungal genetic variability and host and fungal compatibility in this functional diversity. A better understanding of these processes is key to exploit the whole potential of AM fungi for agricultural applications and to increase the nutrient acquisition efficiency and productivity of economically important crop species.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Prairie Cordgrass and its Benefits as a Potential Biofuel

Spartina pectinata Link, also known as prairie cordgrass (PCG), is a warm season, perennial grass native to South Dakota ranging from sizes of 1 to more than 2 meters in height (Boe et al. 2009; Jensen 2006; Johnson et al. 2007). PCG has a broad distribution that reaches all the way into the Arctic Circle of Northern Canada, despite utilizing C_4-based photosynthesis (Johnson et al. 2007; Potter et al. 1995). Using this system makes PCG more efficient at utilizing nitrogen (Sage and Pearcy 1987) and carbon dioxide under limiting conditions, whilst decreasing the effects of photorespiration, which negatively affects C_3-based plant systems (Alberts et al. 2002). PCG also spans the majority of the United States, excluding Alaska, Hawaii, and regions in the southeast and the southwest (Jensen 2006).

In relation to its diverse geographic distribution, PCG can grow in diverse soil conditions that include high levels of moisture (Skinner et al. 2009), well drained lands, and other stresses such as high levels of salinity (Montemayor et al. 2008). According to a study performed by Boe et al. 2009 from 2000 - 2008, populations of PCG were able to produce on average 12.7 $Mg \cdot ha^{-1}$ of biomass at (Boe et al. 2009) and was also able to produce more biomass than switchgrass, another biofuel candidate, can produce under similar conditions (Boe and Lee 2007). Because of these unique abilities, it is crucial for further research to be conducted on this promising biofuel crop.

Due to the ongoing depletion of non-renewable sources of energy such as oil (Aleklett et al. 2010; Bentley 2002; Hirsch 2005), and the ever increasing demand for said resource, initiatives are being made to counteract this dependency. This has led to

the genesis of alternative fuel strategies, one specifically being biofuels. Biofuels are essentially organic material (i.e. biomass) that can be processed into liquid fuels (e.g. ethanol and biodiesel) which can then be used as an additive in oil products, in this case gasoline and diesel fuel. This hybridization of traditional fuel and biofuel can increase octane levels (Cohn et al. 2005; Short and Dickson 2004) and reduce hazardous emissions from the combustion process (Bozbas 2008; Sims et al. 2010).

The two main sources of biofuel, currently, are corn (*Zea mays*) and soybeans (*Glycine max*) (Kim and Dale 2005; Martin 2010). This has led to controversy since these two plants are predominantly used to feed humans and livestock, so the fact that these critical food based resources are being diverted into fuel production brings forth the need for more effective counter strategies. This is where next generation biofuel sources come into play. Because of the previously mentioned beneficial traits, PCG is being considered for use as a potential cellulosic biomass crop along with other notable plant species such as switchgrass (*Panicum virgatum* L.) (Parrish and Fike 2005) and *Miscanthus* × *giganteus* (Heaton et al. 2008). These next generation fuel sources, also known as dedicated energy crops, are predominantly grasses and have no impact on food production for humans. These grasses also grow on marginal land, so the need for diverting precious farm land used for food production is negated, essentially maximizing the potential in an already defined area. Another benefit of growing these crops on marginal land reduces the need for clearing other notable large areas of land, such as forests, which lowers the level of "carbon penalization" that clearing these lands can cause (Dale et al. 2010; Mullins et al. 2010).

Due to the inevitability of improving key traits amongst populations of PCG via breeding strategies (e.g. conventional and/or molecular based) and various transformation techniques, an extensive framework of genetic systems, data sets, and resources must be developed before any further progress can be made.

1.2 Mycorrhizal Endosymbiotic Interactions

Besides the previously mentioned attributes, PCG can form symbiotic relationships with arbuscular mycorrhizal (AM) fungi, a member of the phylum, Glomeromycota. Arbuscular mycorrhiza (AM) symbiosis occurs in ca. 65% of all terrestrial plant species including many economically important crops (e.g. corn, rice, soybean, wheat, etc.) (Smith and Smith 2011) and potential feedstocks such as PCG (unpublished data). This interaction between plant and microbe entails benefits for both organisms where AM fungi will obtain a fraction of the plant's carbon supplies and in return, the plant will receive numerous benefits that can improve the environmental sustainability.

One particular benefit involves the increased uptake of primarily phosphorus and some other nutrients including, nitrogen, sulfur, magnesium, copper, and zinc (Smith and Smith 2011). Other beneficial traits include the increased resistance to both biotic and abiotic stresses such as pathogenicity, drought, high salinity, and heavy metal contamination of soils (Jeffries et al. 2003; Newsham et al. 1995; Singh et al. 2011). It has also been shown that these symbiotic relationships have the potential to carbon sequestration levels (Smith and Read 2008; Treseder and Holden 2013).

Despite our knowledge of these interactions amongst other plants and their significance, the level of research that has been performed on PCG has been rather limited. Previous research has shown that high rates of colonization can occur amongst

PCG varieties native to this region of the United States and that there is the potential for increased levels of biomass development under limiting nutrient conditions. However, due to the high level of genetic variability found within PCG communities, it is still unclear if there is significant variation within this grass species in terms of biomass development, AM colonization rates, and nutrient acquisition.

1.3 The Need for Further Research, Genetic Tools and Systems

Despite the several benefits that PCG exhibits as well as its relationship with AM fungi, further improvements of traits will have to be made in order for this plant species to become a more reliable and efficient biomass feedstock for future producers. This can be achieved through various means including conventional and molecular breeding techniques or through more unorthodox processes such as transgenesis (i.e. gene transfer) (Collard et al. 2005; Gressel 2008; Salgotra et al. 2014; Sreenivasulu et al. 2007; Varshney et al. 2005). Regardless of what methodologies may be used, an extensive understanding and knowledge of the plant's genome and how it expresses its genes under various conditions is crucial for any future advancements.

The key limiting factors for the further analysis and improvement of PCG, at this time, are genetic information, tools, and resources. As of this date, very little genetic based research has been performed on PCG compared to other commercially important cellulosic biomass crops. What genetic research has been performed entails a publication that was released in 2007 discussing the genetic variation in PCG samples found in Minnesota which used amplified fragment length polymorphism techniques (Moncada et al. 2007), a publication released in 2010 discussing a preliminary investigation of the transcriptome of PCG using various tissue systems (Gedye et al. 2010), another

publication released in 2012 discussing the development of simple sequence repeat (SSR) markers for marker assisted breeding of PCG (Gedye et al. 2012), and unpublished research which investigated genetic and cytotypic diversity amongst PCG populations from the Midwest region of the United States (Iowa, Kansas, Minnesota, Nebraska, North Dakota, and South Dakota) utilizing AFLP techniques (Dwire 2010; Monier 2013) and also the construction and initial analysis of a bacterial artificial chromosome (BAC) library developed from the "Red River" cultivar of PCG (Monier 2013).

As mentioned previously, genetic improvements to this plant species can be achieved via molecular breeding techniques including marker assisted selection (MAS). While this approach is now standard in more economically important crops, other less studied plant species, such as potential biomass feedstocks, are only beginning to become targets for these approaches. In order for a series of genome spanning, reliable, and conservative markers to be developed, especially for a plant species that exhibits tetraploid, hexaploid, and octoploid genomes, a wealth of expressed sequence data must be acquired (Kim et al. 2010; Kim et al. 2012).

Besides the earlier stated transcriptome analyses, as of this date, there has been no transcriptome data of PCG, switchgrass, and *Brachypodium* under mycorrhizal and nutrient limiting conditions made available to the public. The objectives of this research will consist of producing, sequencing, and analyzing transcriptome data of PCG, switchgrass, and *Brachypodium* under various nutrient and/or mycorrhizal conditions. From this data, gene/pathway analyses and comparative studies with other plant taxa will be conducted as well as the possibility of generating expressed sequence tags (ESTs) which can be used for marker development.

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CHAPTER 2: MICROBIOME ANALYSIS OF UPPER MIDWEST PRAIRIE CORDGRASS POPULATIONS

2.1 Introduction

Spartina pecinata Link (PCG) is a warm season, perennial grass native to South Dakota. PCG can range from sizes of 1 to 2 meters in height (Johnson et al. 2007) and has a broad distribution across North America, reaching into the Arctic Circle of Northern Canada and down to Texas (Johnson et al. 2007; Potter et al. 1995). Despite its wide environmental distribution, PCG utilizes C4-based photosynthesis and can grow in diverse conditions. These include dry prairie systems of North America, areas with poor soil aeration, waterlogged regions, and soils with elevated salinity (Bonilla-Warford and Zedler 2002; Montemayor et al. 2008; Skinner et al. 2009). PCG can also produce considerable amounts of biomass. Boe et al. (2009) reported from 2000 to 2008, populations of PCG could produce an average 12.7 $Mg \cdot ha^{-1}$ of biomass. Due to these aforementioned traits, PCG has been considered as a next-generation cellulosic feedstock.

The plant microbiome is a diverse and complex system of microbiota that interact with a plant host. These interactions can range from mutualism, commensalism, or parasitism (Johnson et al. 1997; Paszkowski 2006; Redman et al. 2001). Myriads of studies have been conducted on understanding the microbiome of various plants and the leaf, root, phyllo-, and rhizospheres. Many of these reports indicate microbial traits that are beneficial for the plant (Lugtenberg and Kamilova 2009; Porras-Alfaro and Bayman 2011; Smith and Smith 2011). These beneficial traits include, improved nutrient acquisition from the soil, plant growth hormone production, and increased tolerance to abiotic and biotic stresses (Ahmad et al. 2008; Bashan and De-Bashan 2005). Microorganisms that exhibit these growth promoting traits are commonly referred to as

plant growth promoting microorganisms (PGPM). Due to the complexity and infancy of this research, our knowledge about PGPM is limited to studies with individual isolates, usually under laboratory conditions (Bulgarelli et al. 2013). This limitation can also impede our understanding of how entire microbial communities can affect the growth of plants.

PCG is no exception to these phenomena. Despite PCG's promising attributes, little is known about its interactions to PGPM. Prior unpublished data has shown that associations with bacteria that are able to fix gaseous nitrogen can occur. Previous studies have also shown that when PCG is inoculated with other beneficial microbial symbionts, such as arbuscular mycorrhizal fungi (AMF), a significant increase in biomass yield potential can be observed (Monier et al. *unpublished*). Additionally, Liepold (2013) used denaturing gradient gel electrophoresis techniques to show that natural populations of PCG can form associations with a variety of AMF strains.

Similar to classic ecological research, microbial communities in plants can change in terms of diversity and richness. These changes can be attributed to a variety of factors including temporal (e.g. seasonal patterns), geospatial, and environmental changes. Despite PCG's ability to grow in a wide variety of environmental locations, no research has been conducted to study the alpha- and beta-diversity in response to different environmental conditions.

With the rise of high-throughput sequencing technology, researching microbial community structure in plants has become more accessible. By sequencing the PCR amplicons of hypervariable genes (e.g. 16S/18S rRNA), we can achieve a much higher resolution of overall community composition from many branches of life. Therefore, we

propose the following objectives: (1) determine the microbiome composition of PCG in above- and below-ground tissue using primers that will amplify prokaryotic, eukaryotic, and AMF taxa; (2) determine alpha- and beta-diversity of PCG samples collected in the Upper Midwest.

2.2 Materials and Methods

2.2.1 Sample Collection

Prairie cordgrass samples were collected from 65 locations across several states (South Dakota, Iowa, Minnesota, and Nebraska) (Figure 2.1). Sample locations were based off of prior survey work to determine prairie cordgrass diversity (Dwire 2010). Sample locations were only visited once during the month of June in 2015. A GPS (global positioning system) unit was used to mark the longitude and latitude coordinates of each site. Additionally, brief notes were taken about each site in terms of environmental properties (Table 2.1).

Above and below-ground tissue was collected from each sample site using one plant. For above-ground tissue, only leaf material was collected. Below-ground tissue collection was a mixture between root and rhizome material. Once tissue was excised, samples were placed on ice and returned to the lab for additional processing. At the lab, residual soil material on the below-ground material was washed off using tap water. After cleaning, all tissue samples were flash frozen in liquid nitrogen and stored at -80° C until further analysis.

2.2.2 DNA Extraction

A modified DNA extraction protocol from Doyle (1987) was used for this process. Briefly, approximately 500 milligrams of either above- or below-ground tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. 500 µl of CTAB extraction buffer (2.5 M polyvinylpyrrolidone, 1.42 M sodium chloride, 0.5 M ethylenediaminetetraacetic acid, 0.1 M tris -hydrochloride, 0.055 M cetrimonium bromide) was added to each sample and incubated at 60° C for 20 minutes. Next, samples

were centrifuged at 10,000 RPM for two minutes to pellet cellular debris. Supernatant was transferred to a new tube containing 400 µl of chloroform:isoamyl alcohol (24:1, v:v) and centrifuged at 10,000 RPM for five minutes. After centrifugation, the aqueous top layer was removed and added to 300 µl of isopropanol for DNA precipitation. DNA was pelletized by centrifugation at 10,000 RPM. Finally, the DNA pellet was washed with 200 µl of 70% ethanol, dried, and resuspended in 75 µl of TE buffer (10 mM tris-hydrochloride, 1 mM ethylenediaminetetraacetic acid). To degrade RNA, DNA samples were treated each with 1 unit of RNase ONE™ Ribonuclease (Promega, Madison, WI). To check the quality of DNA, an aliquot of each sample was ran on a 1.2% (w/v) agarose gel with TAE buffer (40 mM tris, 20 mM acetic acid, 1 mM ethylenediaminetetraacetic acid). DNA quantity was determined using a ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE)

2.2.3 Amplicon Sequencing

Samples were sent out to the W.M Keck Center for Comparative and Functional Genomics (University of Illinois) for sequencing. Samples were sequenced using Illumina MiSeq V3 technology. Library construction and amplicon generation was performed using the Fluidigm platform. To obtain a high resolution of the prairie cordgrass microbiome, five primer pairs were used. These primer pairs spanned a wide range of microbiota including potential targets in prokaryotic and eukaryotic taxa (Table 2.2).

2.2.4 Data QC, Alignment, and Analysis

Demultiplexing and primer sorting was also performed at the W.M. Keck Center for Comparative and Functional Genomics. Sequence quality checks, BLAST analysis,

and OTU generation were performed using the program *MOTHUR* (version 1.38). Maarj*AM*, SILVA, and UNITE databases were used for the alignment steps. The Maarj*AM* database was used to align the sequences generated by the AM fungal primers (AMV4.5NF/AMDGR and NS31/AML2) (Öpik et al. 2010). The SILVA database (v128) was used for the prokaryotic primer set (F357/R926) and the UNITE database was used for the eukaryotic primer set (ITS1F/ITS4R).

Specified parameters for each step was performed using the MiSeq standard operating procedure (SOP) pipeline for *MOTHUR* (Kozich et al. 2013). Beforehand, concatenation of the general eukaryotic and prokaryotic primer set was performed using IM-TORNADO (version 2.0.3.3) (Jeraldo et al. 2014). This was due to the actual target amplicon exceeding 600 bp which caused no overlap in the reads. OTU alpha and beta diversity analyses along with other statistical measures were performed using the program, *R* (version 3.4.2), along with the *R* package, *vegan* (version 2.5.2) (Oksanen et al. 2007; R Core Team 2017).

2.3 Results

2.3.1 Primer metrics

After sample processing and sequencing, over five and ten million total reads were produced for both above- and below-ground tissue samples of PCG, respectively (Table 2.2). After separating the reads by primer set, we noticed some disparities. For the above-tissue, over 95 percent of the total reads were generated using the prokaryotic primers (F357/R926), while over half of the sequences generated with the below-ground PCG tissue were produced using the AMF primers, AMV4.5NF/AMDGR. In both tissue types, the diazotroph primers (F2/R6) failed to produce a significant number of reads (Table 2.4), and were therefore not further analyzed.

Once the reads were processed in *MOTHUR*, only the prokaryotic primer pair remained usable for above-ground tissue. In the below-ground tissue samples, four out of the five primer pairs passed quality control. Similar to the prior metrics, the diazotroph primer set failed quality control and was not used for any additional analysis (Table 2.5). Due to the failed quality checks of some of the primer pairs and tissue sample combinations, only five out of the possible ten sample sets could be analyzed. These included the F357/R926 primer set targeting the (1) above- and (2) below-ground PCG tissue, (3) the ITS1F/ITS4R primer set for below-ground tissue, and (4, 5) the AMF primers (AMV4.5NF/AMDGR and NS31/AML2) for below-ground tissue.

2.3.2 Alpha diversity

Of the five primer pair/tissue combinations, OTUs were generated. The prokaryotic primer pair produced the highest amount of OTUs for both tissue types. Both

AMF primer pairs produced the lowest amount, with NS31/AML2 generating 100 entities (Table 2.6).

Shannon diversity and Chao1 richness indexes were produced as measures for the alpha diversity with the remaining primer pairs. When analyzing these indexes for each primer/tissue combination, they share a similar positive relationship with the number of OTUs generated. Based on these findings, the more OTUs that are present, Shannon diversity and Chao1 richness indexes are coincidentally higher (Table 2.7).

To determine if there was any significant variability within each primer/tissue combination for Shannon or Chao1 metrics, a series of ANOVAs were performed. Variance was determined for the factors, “state” and “location”. Significant differences were detected for both state and location for Shannon diversity. These variances were only found in the prokaryotic and AMF (only NS31/AML2) microbiomes in the below-ground tissue (Table 2.8). Likewise, Chao1 richness varied significantly for both state and location for the prokaryotic and AMF microbiomes (Table 2.9).

Next, pairwise comparisons were conducted to see which specific states or environmental locations differed for the prokaryotic microbiome. Both Shannon and Chao1 metrics were significantly higher in the samples collected from Iowa compared to samples from either Minnesota or South Dakota, but not Nebraska (Figure 2.2). Additionally, samples collected from riparian areas had significantly lower Shannon and Chao1 metrics when compared to the other environments (Figure 2.2).

The AMF microbiome was also analyzed for pairwise comparisons. Significant variability was only observed for environmental locations. For the AMV4.5NF/AMDGR primer set, Chao1 richness was only significantly lower for riparian areas when compared

to roadside ditches (Figure 2.3). Likewise, the NS31/AML2 primer set showed decreased Chao1 richness but also decreased Shannon diversity for riparian areas when compared to the other environmental locations including roadside ditches, natural grasslands, and experimental test plots (Figure 2.4).

2.3.3 Taxonomic abundance

After OTUs were taxonomically classified, their proportional abundances were calculated for each sample. Each primer pair/tissue combination was analyzed at the phyla, class, and species level. First, the prokaryotic microbiome was analyzed for above-ground tissue. At the phylum level, a majority of the OTUs were classified as proteobacteria. A significant number of samples also contained entities belonging to Actinobacteria and Bacteroidetes (Figure 2.5). At the class level, Alphaproteobacteria had the highest abundances. Additionally, Actinobacteria and Gammaproteobacteria had significant numbers (Figure 2.6). Abundances at the species level were highly diverse. To simplify the analyses, only the top 10 percent of abundant species were examined. The remaining samples were partitioned into a "other" category. In contrast to the "other" category, *Sphingomonas* sp., and *Pseudomonas* sp. were prevalent in many of the samples (Figure 2.7).

Conversely, the prokaryotic microbiome in below-ground PCG tissue was also analyzed. The phylum level had a much broader range, with many samples containing high abundances related to the phyla, Proteobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, and Acidobacteria (Figure 2.8). The class level also reflected a broad taxonomic range. This included key classes of Alpha-, Beta-, Gamma-, and Deltaproteobacteria. Also, Actinobacteria, Cytophagia, Flavobacteriia, Thermoleophillia,

and Sphingobacteriia were found in a majority of analyzed samples (Figure 2.9). Like the above-ground prokaryotic microbiota, only the top 10 percent of species were analyzed. The lower 90 percent of hits were categorized in the category, “others”. Besides a majority of the taxonomic hits belonging to the “others” category, species belonging to the *Streptomyces*, *Rhodoplanes*, *Flavobacterium*, *Pseudomonas*, *Devosia,* and *Methylibium* genera were also identified (Figure 2.10).

Next, the eukaryotic primers (ITS1F/ITS4R) for below ground PCG tissue were analyzed. At the phylum level, only a few identifications were detected. A majority of these samples had hits belonging to the Ascomycota, Basidiomycota, and Glomeromycota. Some hits also came back as “unclassified” indicating poor sequence alignment to the BLAST database (Figure 2.11). At the class level only the top 25 percent of classes were analyzed. Leotiomycetes, Agaricomycetes, Dothideomycetes were among the most prevalent classes. It should also be noted that the “others” category did contain samples that were identified as “unclassified” (Figure 2.12). Likewise, species hits were only analyzed for the upper quartile of total abundance. In contrast to the prior observations, taxonomic hits were scattered and did not appear to have any prominent species for all samples (Figure 2.13).

Finally, the AMF microbiota were analyzed. For these analyses, only the species level of taxonomy was of importance due to the limited taxonomic range of AMF. For the AMV4.5NF/AMDGR primer set, a few prominent species were identified. *Glomus hoi*, *Rhizophagus intraradices,* and *Rhizophagus irregularis* were found in a majority of samples. Additionally, many samples registered hits for a plethora of *Glomus* sp. taxonomic IDs. These IDs were classified into their own category, “*Glomus* sp.” to avoid

overcrowding of the figure (Figure 2.14). The NS31/AML2 primer set also had a lot of hits for various *Glomus* sp. IDs. In contrast, this primer set identified *Glomus indicum* in some samples. Similar to the prior AMF primer pair, *Rhizophagus irregularis* and *Rhizophagus intraradices* were identified, albeit at lower levels (Figure 2.15).

2.3.4 Beta diversity

To determine if there was any significant shifts in community structure by previously mentioned environmental and locational variables, beta diversity was performed. This was accomplished by using PERMANOVA tests with 1,000 replicate permutations for Bray-Curtis dissimilarity indexes on state and location factors. Significant variances for both state and location were identified in the prokaryotic and AMF primer pairs (Table 2.10).

To determine which factor levels had significant community shifts, pairwise comparisons were performed by running PERMANOVA tests for each possible comparison. To adjust for multiple-testing corrections, FDR was performed. Above ground tissue analysis for the prokaryotic microbiome showed that community shifts were present when comparing samples from South Dakota to either Iowa or Nebraska. However, when adjustments for multiple testing corrections were implemented, the probability for this alternative hypothesis decreased (Table 2.11).

Likewise, comparisons were also made for the environmental location of the collection site. For the primers that were analyzed, prokaryotic and AMF communities in riparian areas seemed to significantly differ when compared to the other environmental locations. While this was observed in each primer pair, only the AMF primers showed significant changes (Table 2.12). This was also made apparent with PcoA. Riparian areas

were shown to be more clustered when compared to the other locations (Figure 2.16). Additionally, abundances were also visualized for these two primer pairs with samples being partitioned by location instead state. When grouping the samples in this scheme, an overall reduction in multiple types of AMF taxa is observed (Figure 2.17, Figure 2.18).

2.4 Discussion

The data presented here represents the first high-throughput sequencing-based amplicon survey of microbial diversity in PCG. By using multiple primer sets, we were able to gain an insight into prokaryotic and fungal diversity in above- and below-ground PCG tissue. While we could analyze microbial diversity for both PCG tissues, the diazotroph primer set failed to produce any reliable results. Only 13 PCG samples came back with a minimal number of reads. After QC and rarefication, none of the original reads passed for either tissue type. The diazotroph primer pair (F2/R6) was chosen based off of *in silico* analysis performed by Gaby and Buckley (2012). Based off their analysis, F2/R6 was found to be one of the best primer pairs compared to other available primer sets at that time. Recently, Angel et al. (2018) showed that this primer pair has a tendency to discriminate against various diazotrophs within environmental samples. Coincidentally, Gaby and Buckley (2015) have also shown this discriminatory nature when applying this primer set to environmental samples.

Alpha diversity analysis revealed significant differences for Shannon diversity and Chao1 richness for multiple primer sets. This was observed for both state and environmental location. Prior research has shown similar geographical trends. Coleman-Derr et al. (2016) observed shifts in Shannon diversity for both prokaryotic and eukaryotic communities in relation to geographical distance of *Agave* plants. Ma et al. (2017) also examined distinct biogeographical trends in microbial communities across Eastern China. Although this was observed in the prokaryotic PCG root endosphere, this could be due to some underlying factor. For example, Fierer and Jackson (2006) showed differences in microbial diversity via spatial distance, but was related more to the overall

pH of the soil. More instances of alpha diversity shifts were observed for the environmental location of the sample for both prokaryotic and AMF communities in below-ground tissue. In each of these observations, riparian areas displayed lower Shannon diversity and Chao1 richness when compared to other locations. This trend was also reported in Lin et al. (2014) with upland and lowland bacterial communities of *Casuarina*. Fan et al. (2016) also showed decreases in alpha diversity of microbial communities taken at riparian areas compared to other locations. The most likely causes in these instances were due to changes in nutritional composition, pH, and heavy metal contaminants.

Beta diversity was also analyzed. PERMANOVA tests show that significant variations in riparian areas compared to other sampling locations was found in AMF communities. Riparian and lowland locations have been shown to shift bacterial and fungal community structure. Bonito et al. (2014) reported significant fungal endophyte community changes in *Quercus, Pinus, Populus* seedlings in lowland areas compared to high land samples. Vasconcellos et al. (2013) also showed significant fungal and bacterial shifts in semideciduous forest plants based on their proximity to riparian zones. Beauchamp et al. (2006) showed that certain AMF can fluctuate in abundance based on moisture content and distance from an active river channel of the sampling site in *Populis-Salix* stands in a semiarid riparian ecosystem. This shift in community, decrease in diversity, and richness can be attributed to the fact that AMF respond negatively to increased levels of soil moisture (Stevens and Peterson 1996; Turner and Friese 1998). Likewise, Deepika and Kothamasi (2015) showed similar patterns in AMF diversity

reduction and shift to fewer, more prominent AMF strains in sorghum and their relationship to soil moisture.

The main goal of this project was to gain preliminary insights into the actual composition of the PCG microbiome. We determined proportional abundances for prokaryotic, eukaryotic, and AMF microbiota for either above- or below-ground tissue types. The prokaryotic microbiome for the PCG phyllosphere was analyzed targeting the 16S rDNA gene. Above-ground PCG tissue contained several phyla of proteobacteria. The taxa belonged mainly to the alpha- and gammaproteobacteria classes. At the genus and species level, we identified mainly *Sphingomonas* and *Pseudomonas* species. These findings coincide with microbiome studies of the leaf endosphere in other grasses. Ren et al. (2015) also reported over 90% of the leaf microbiome in rice belongs to classes within the proteobacteria, mainly alpha- and gammaproteobacteria. Wallace et al. (2018) also showed similar findings in maize where 80% of the entire leaf microbiome consists of mainly alphaproteobacteria and some gammaproteobacteria. Additionally, this trend is observed in wheat, switchgrass, and sugarcane (Gdanetz and Trail 2017; Hamonts et al. 2018; Xia et al. 2013). Hamonts et al. (2018) and Wagner et al. (2016) also showed high levels of mainly *Sphingomonas* species but not *Pseudomonas* in sugarcane and perennial wild mustard, respectively. *Sphingomonas* and *Pseudomonas* strains were identified in a microbiome study in Arabidopsis (Ritpitakphong et al. 2016). This paper also showed that strains belonging to these genera have PGP qualities, in which protected the plant against fungal pathogens. *Sphingomonas* and *Pseudomonas* strains have shown PGP qualities in other plants. Khan et al. (2017) showed that *Sphingomonas* strains can aid tomato plants in salinity resistance. Innerebner et al. (2011) reported that *Shingomonas*

can protect Arabidopsis plants from known plant pathogens including *Pseudomonas syringae*. *Pseudomonas* strains have been shown to promote growth in chickpea plants, sorghum, and winter oilseed rape via phosphate solubilization, and indole acetic acid production (Goswami et al. 2013; Hameeda et al. 2007; Lally et al. 2017).

The prokaryotic microbiome of the PCG below-ground tissue was similar in phyla and class composition. We observed alpha-, beta-, gamma-, and deltaproteobacteria, along with Actinobacteria, Cytophagia, Flavobacteriia, Thermoleophillia, and Sphingobacteriia taxa. Similar taxonomic profiles have been prevalently shown in the roots and rhizosphere of maize (Niu et al. 2017; Peiffer et al. 2013), rice (Edwards et al. 2015), sorghum (Xu et al. 2018), and wheat (Rascovan et al. 2016). *Streptomyces*, *Rhodoplanes*, *Flavobacterium, Pseudomonas, Devosia,* and *Methylibium* were identified at the genus/species level. Yang et al. (2017) and Walters et al. (2018) reported a similar microbiome profile in maize. *Streptomyces*, *Pseudomonas*, and *Methylibium* have also been identified in Arabidopsis (Lundberg et al. 2012; Schlaeppi et al. 2014), sorghum (Xu et al. 2018), and maize (Niu et al. 2017). Similar to their above-ground counterparts, *Streptomyces* and *Pseudomonas* species have been shown to have PGP qualities. This has been observed in sorghum (Xu et al. 2018), and *Achyranthes aspera* L. (Devi et al. 2017). Santana et al. (2016) has shown that *Rhodoplanes, Methylibium*, and *Devosia* are predominantly found in the shrub, *Baccharis dracunculifolia* DC. and are known to be important for nitrogen fixation (Rivas et al. 2002; Werner and Newton 2005). Youseif (2018) reported that strains of *Flavobacterium* can aid in PGP via increased IAA production.

Next, the eukaryotic microbiome of below-ground PCG tissue was analyzed. Similar to the prokaryotic results, a wide range of taxa were identified. The predominant taxa observed reside within the Ascomycota, Basidiomycota, and Glomeromycota phyla. From these phyla, Leotiomycetes, Agaricomycetes, Dothideomycetes, Sordariomycetes, and Glomeromycetes classes are the most prevalent. Similar profiles were identified in wheat (Gdanetz and Trail 2017). In contrast to the other microbiomes, no prevalent taxa at the genus and species level were identified across all the samples. *Microdochium bolleyi* and *Sclerostagonospora phragmiticola* were found in several samples, regardless of geographical distance. *S. phragmiticola* has been identified as a fungal endophyte in common reed that is prevalent in root endosphere under salt stress and can elicit PGP qualities in rice seedlings (Soares et al. 2016). Conversely, *M. bolleyi* has been shown to be somewhat pathogenic in nature and commonly forms associations with various cereal crops (Hannukkala and Koponen 1988). Despite its pathogenicity, *M. bolleyi* can prevent infection from *Fusarium* species (Reinecke et al. 1979) and *Gaeumannomyces graminis vartritici* (Kirk and Deacon 1987) in wheat.

Finally, the AMF microbiome was analyzed. For both primer pairs analyzed, *Glomus hoi, Rhizophagus irregularis, Rhizophagus intraradices,* and *Glomus indicum* were found to be abundant in many of the samples tested. In prior research, we have identified similar community patterns in over 20 PCG samples taken from Brookings County in South Dakota (Liepold 2013). Additionally, we have found that these abundant taxa are prevalent across a wider geographical area across the Upper Midwest compared to the initial Brookings report. While our findings match these Liepold's prior results, our data provides a higher resolution of the AMF community with the inclusion of less

abundant taxa. One of reasons for this is that we have used more up-to-date primers for this analysis (Van Geel et al. 2014). The inclusion of the AMV4.5NF/AMDGR and NS31/AML2 sets has been shown to amplify a wider range of AMF strains compared to Liepold's AM1/NS31 set. Second, the shift to high-throughput, amplicon sequencing has allowed for the expedited analysis of even more samples compared to the error-prone and time-consuming nature of denaturing gradient gel electrophoresis techniques.

In conclusion, we have gained further insight into the microbial community composition of PCG in the Upper Midwest. This multi-faceted biome approach has identified key players of the prokaryotic, eukaryotic, and AMF communities, while corroborating prior AMF community research. We have also shown significant environmental shifts in community diversity, richness, and structure in prokaryotic and AMF microbiomes. While we did perform some analysis into alpha- and beta-diversity, further investigations about how environmental conditions affect community structure should be conducted. This could include collecting more information about the physico-chemical properties of the sampling locations. Additionally, investigating the microbiome at different seasonal times could provide insight into potential temporal variability of microbial communities.

2.5 Figures

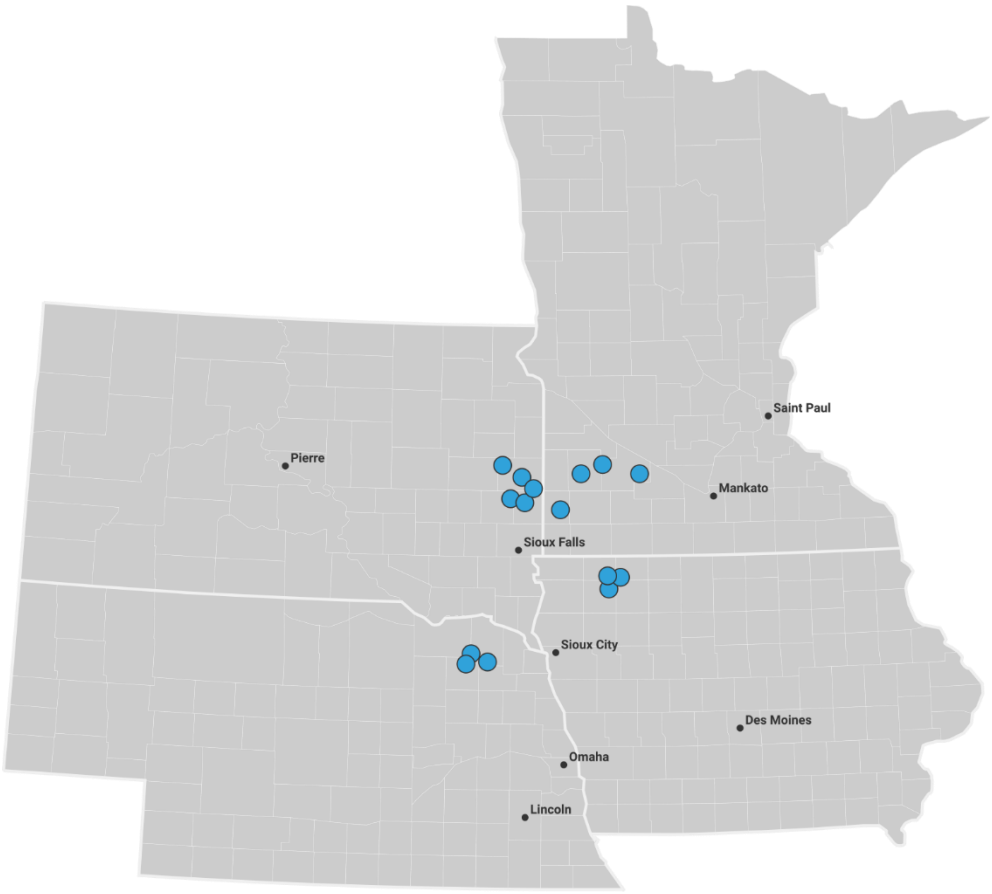

Figure 2.1. Sampling locations of PCG. Approximate site locations (blue dots) of PCG samples were taken across 4 states including Iowa, Minnesota, Nebraska, and South Dakota.

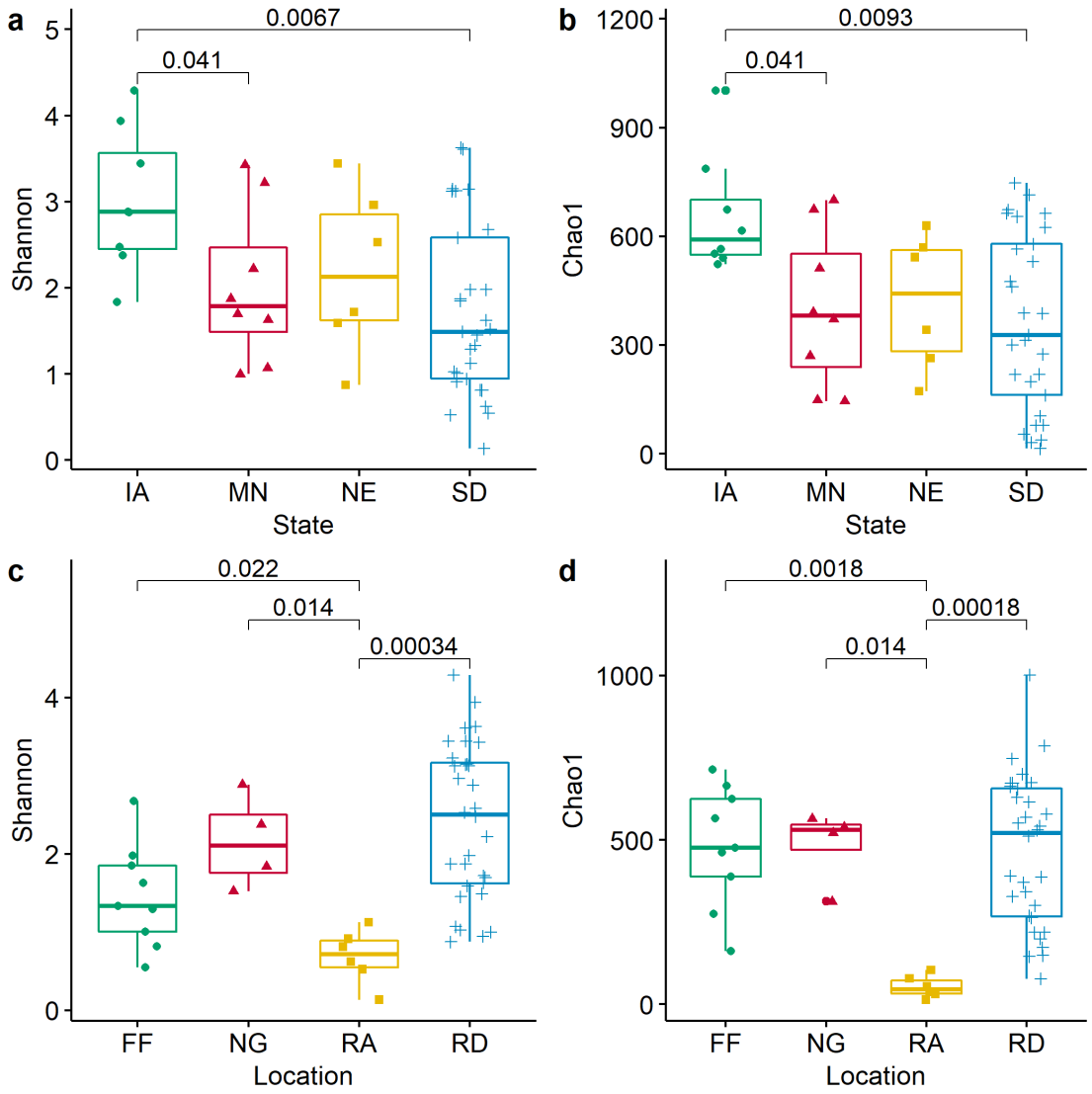

Figure 2.2. Alpha diversity in below-ground PCG tissue using F357/R926 primers. Boxplots for alpha diversity metrics of Shannon Diversity index (A and C) and Chao1 richness index (B and D) for state (A and B) and Location (C and D) factors. Comparisons in each boxplot denote p-values for pairwise comparisons ≤ 0.05. State levels are denoted as Iowa (IA), Minnesota (MN), Nebraska (NE), and South Dakota (SD). Location levels are denoted as Felt Farm experimental plot (FF), natural grassland (NG), riparian area (RA), or roadside ditch (RD).

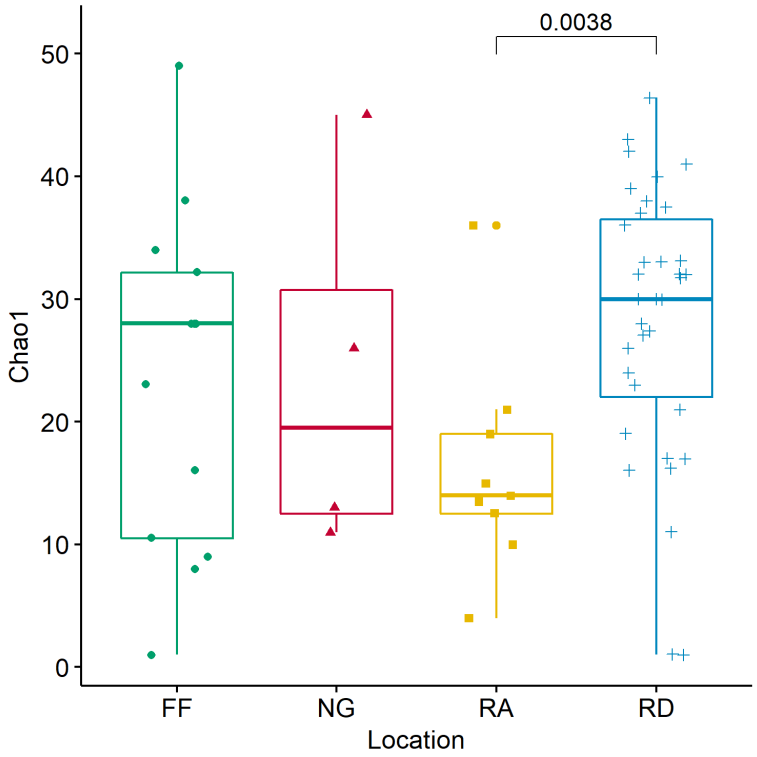

Figure 2.3. Alpha diversity in below-ground PCG tissue using AMV4.5NF/AMDGR primers. Boxplot for Chao1 richness index. Comparisons in each boxplot denote p-values for pairwise comparisons ≤ 0.05. Location levels are denoted as Felt Farm experimental plot (FF), natural grassland (NG), riparian area (RA), or roadside ditch (RD).

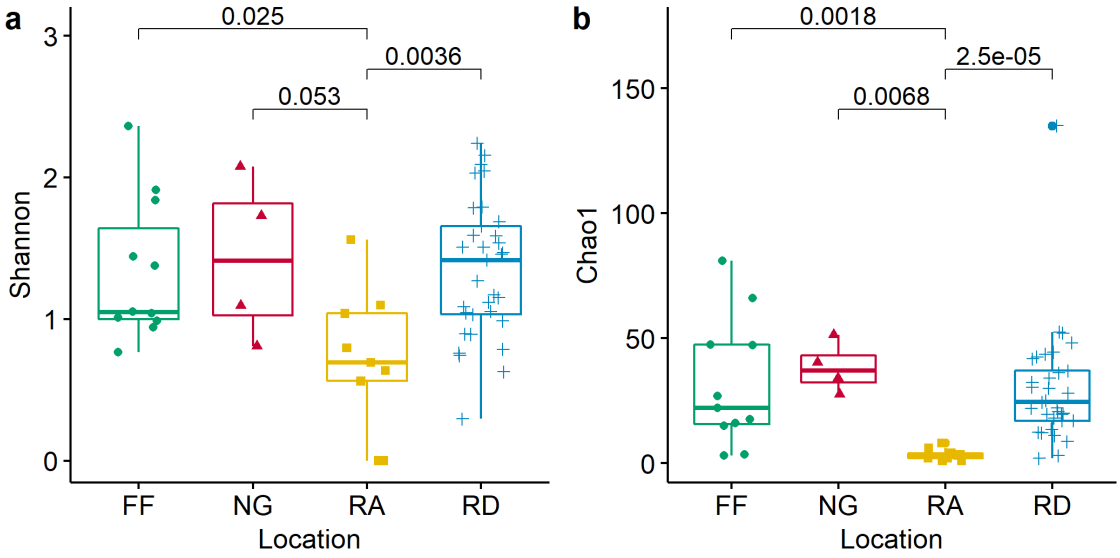

Figure 2.4. Alpha diversity in below-ground PCG tissue using NS31/AML2 primers. Boxplots for Shannon diversity (A) and Chao1 richness indexes (B). Comparisons in each boxplot denote p-values for pairwise comparisons ≤ 0.05. Location levels are denoted as Felt Farm experimental plot (FF), natural grassland (NG), riparian area (RA), or roadside ditch (RD).

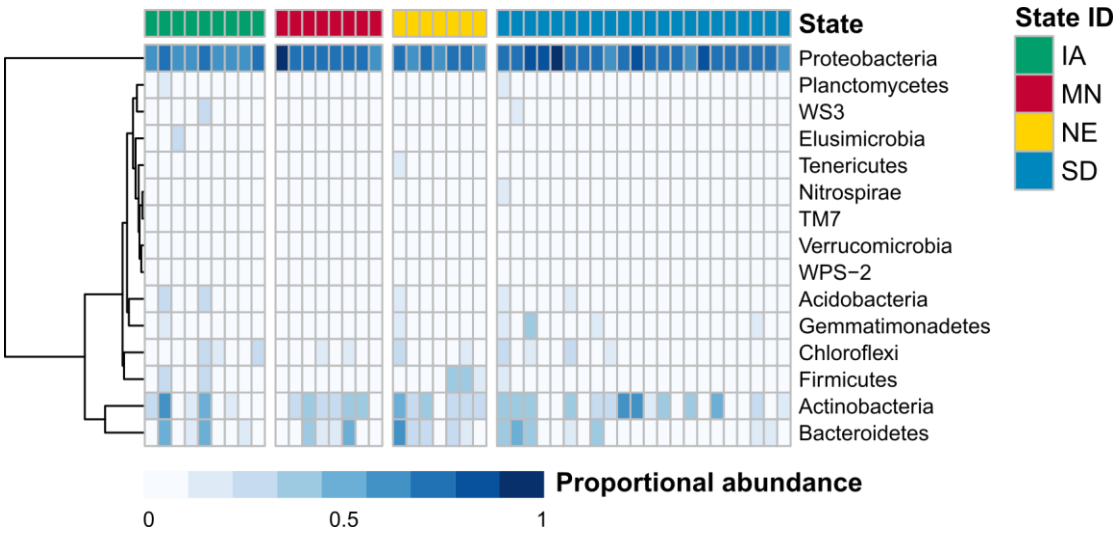

Figure 2.5. Abundance distributions of prokaryotic phyla in above-ground PCG tissue using the F357/R926 primer set. Proportional abundances are displayed in a heatmap where color intensity correlates to abundance values. Rows indicate prokaryotic phyla and columns denote individual PCG samples. Samples are partitioned by the state where they were collected from. State IDs signify either Iowa (IA), Minnesota (MN), Nebraska (NE), or South Dakota (SD). Rows are clustered via complete-linkage clustering.

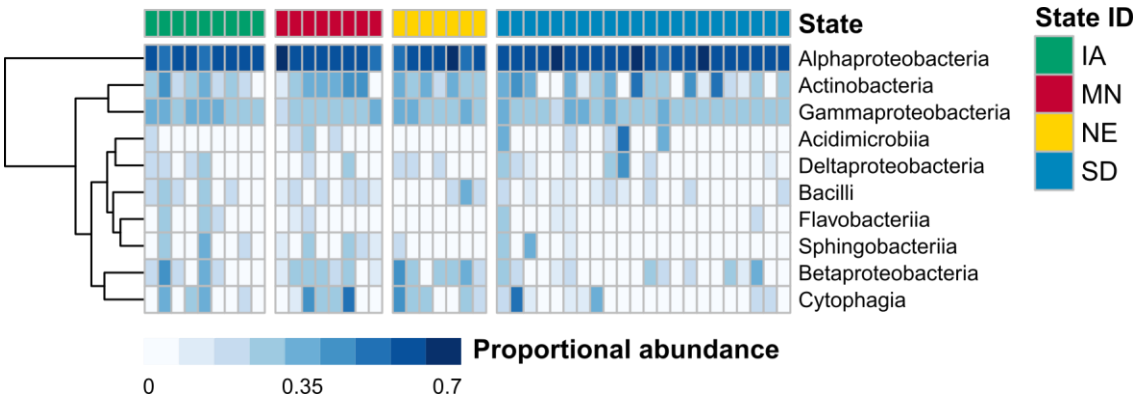

Figure 2.6. Abundance distributions of prokaryotic classes in above-ground PCG tissue using the F357/R926 primer set. Proportional abundances are displayed in a heatmap where color intensity correlates to abundance values. Rows indicate prokaryotic classes and columns denote individual PCG samples. Samples are partitioned by the state where they were collected from. State IDs signify either Iowa (IA), Minnesota (MN), Nebraska (NE), or South Dakota (SD). Rows are clustered via complete-linkage clustering.

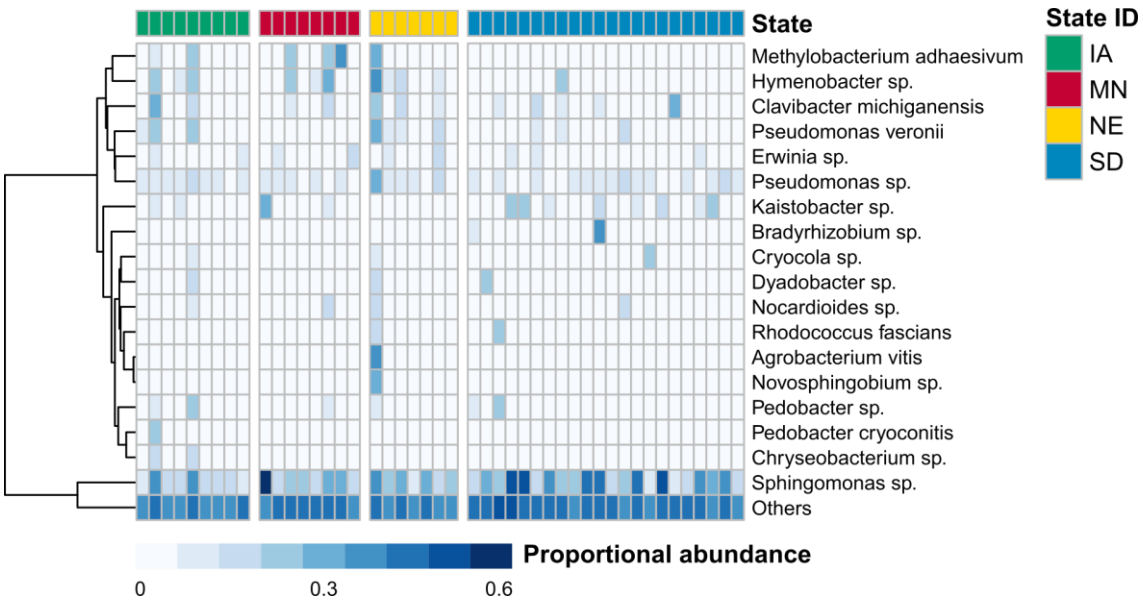

Figure 2.7. Abundance distributions of the top 10 percent prokaryotic species in above-ground PCG tissue using the F357/R926 primer set. Proportional abundances are displayed in a heatmap where color intensity correlates to abundance values. Rows indicate prokaryotic species and columns denote individual PCG samples. Samples are partitioned by the state where they were collected from. State IDs signify either Iowa (IA), Minnesota (MN), Nebraska (NE), or South Dakota (SD). Rows are clustered via complete-linkage clustering. Species' abundance that is < 10% are partitioned into the category "Others".

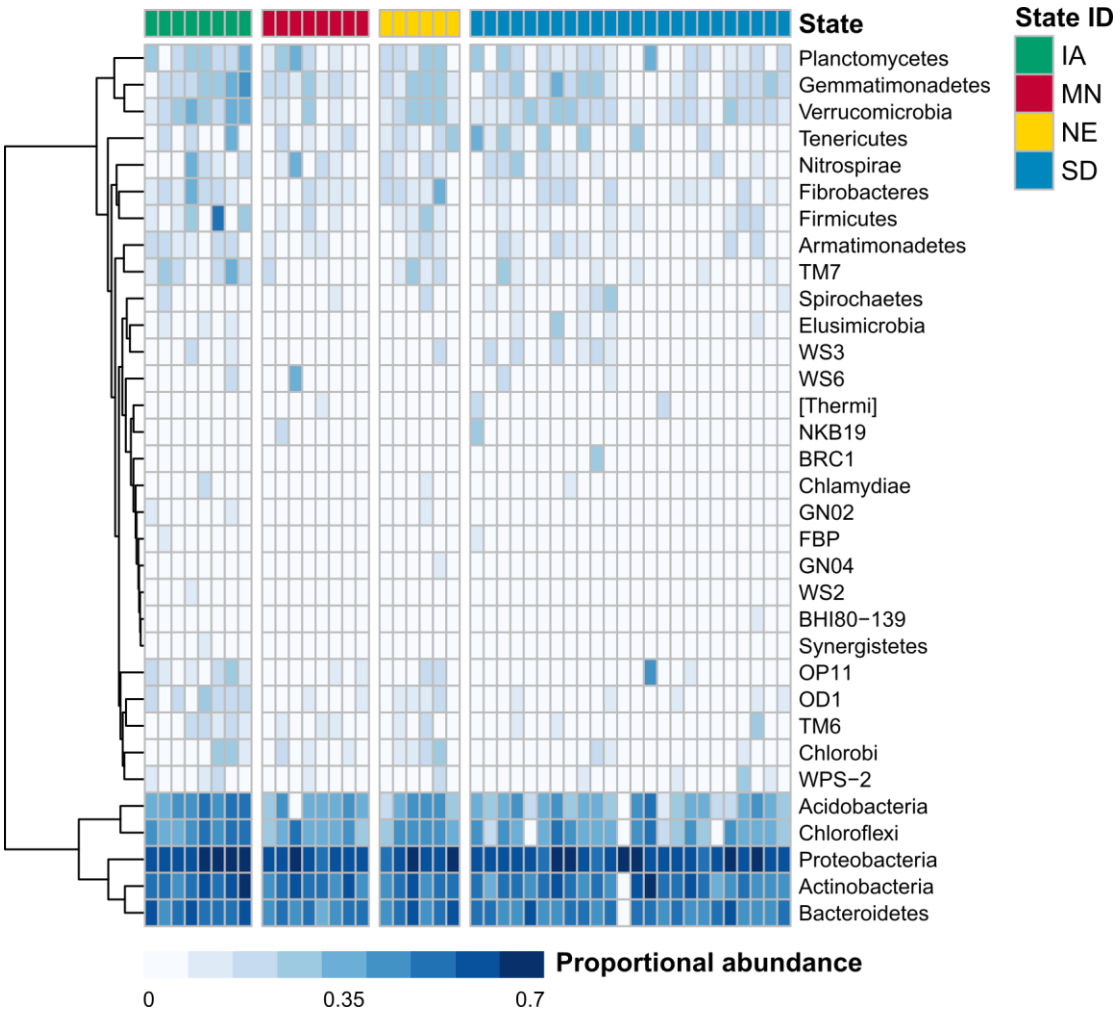

Figure 2.8. Abundance distributions of prokaryotic phyla in below-ground PCG tissue using the F357/R926 primer set. Proportional abundances are displayed in a heatmap where color intensity correlates to abundance values. Rows indicate prokaryotic phyla and columns denote individual PCG samples. Samples are partitioned by the state where they were collected from. State IDs signify either Iowa (IA), Minnesota (MN), Nebraska (NE), or South Dakota (SD). Rows are clustered via complete-linkage clustering.

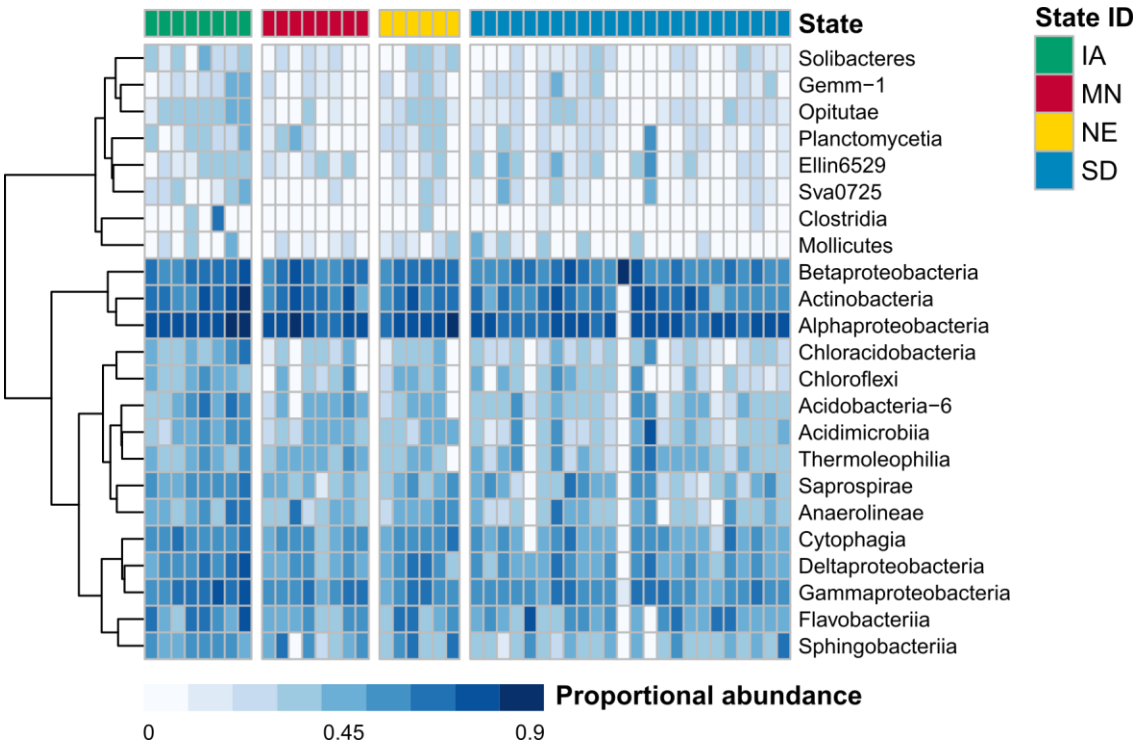

Figure 2.9. Abundance distributions of prokaryotic classes in below-ground PCG tissue using the F357/R926 primer set. Proportional abundances are displayed in a heatmap where color intensity correlates to abundance values. Rows indicate prokaryotic classes and columns denote individual PCG samples. Samples are partitioned by the state where they were collected from. State IDs signify either Iowa (IA), Minnesota (MN), Nebraska (NE), or South Dakota (SD). Rows are clustered via complete-linkage clustering.

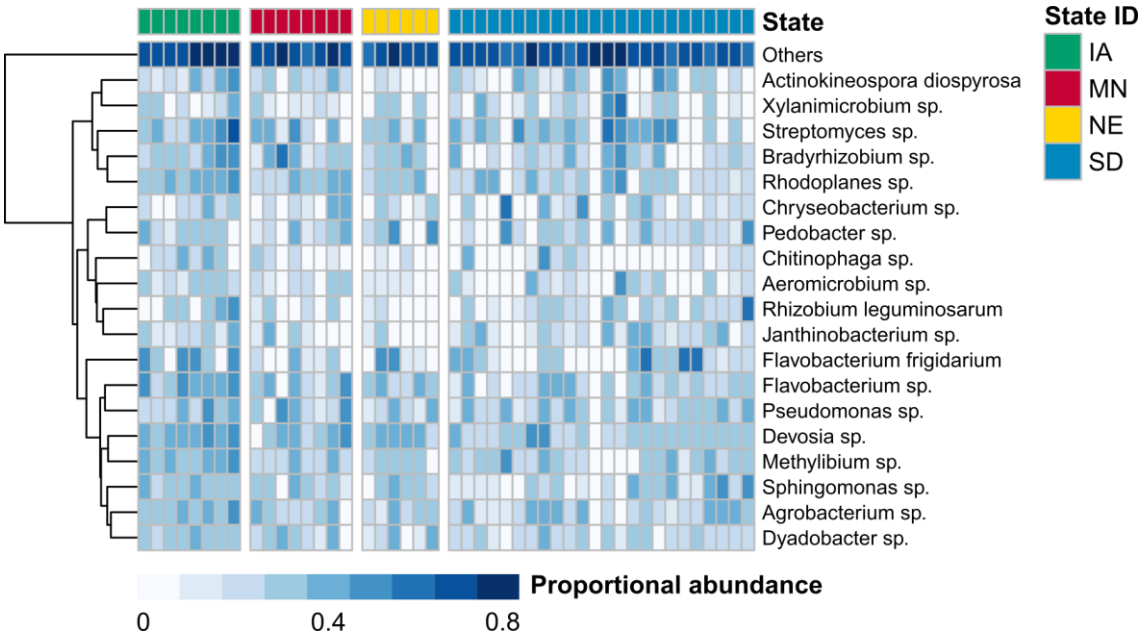

Figure 2.10. Abundance distributions of the top 10 percent prokaryotic species in below-ground PCG tissue using the F357/R926 primer set. Proportional abundances are displayed in a heatmap where color intensity correlates to abundance values. Rows indicate prokaryotic species and columns denote individual PCG samples. Samples are partitioned by the state where they were collected from. State IDs signify either Iowa (IA), Minnesota (MN), Nebraska (NE), or South Dakota (SD). Rows are clustered via complete-linkage clustering. Species' abundance that is < 10% are partitioned into the category "Others".

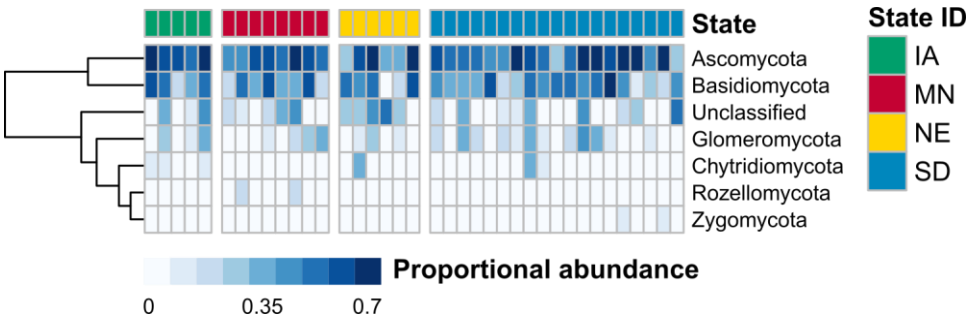

Figure 2.11. Abundance distributions of eukaryotic phyla in below-ground PCG tissue using the ITS1F/ITS4R primer set. Proportional abundances are displayed in a heatmap where color intensity correlates to abundance values. Rows indicate eukaryotic phyla and columns denote individual PCG samples. Samples are partitioned by the state where they were collected from. State IDs signify either Iowa (IA), Minnesota (MN), Nebraska (NE), or South Dakota (SD). Rows are clustered via complete-linkage clustering.

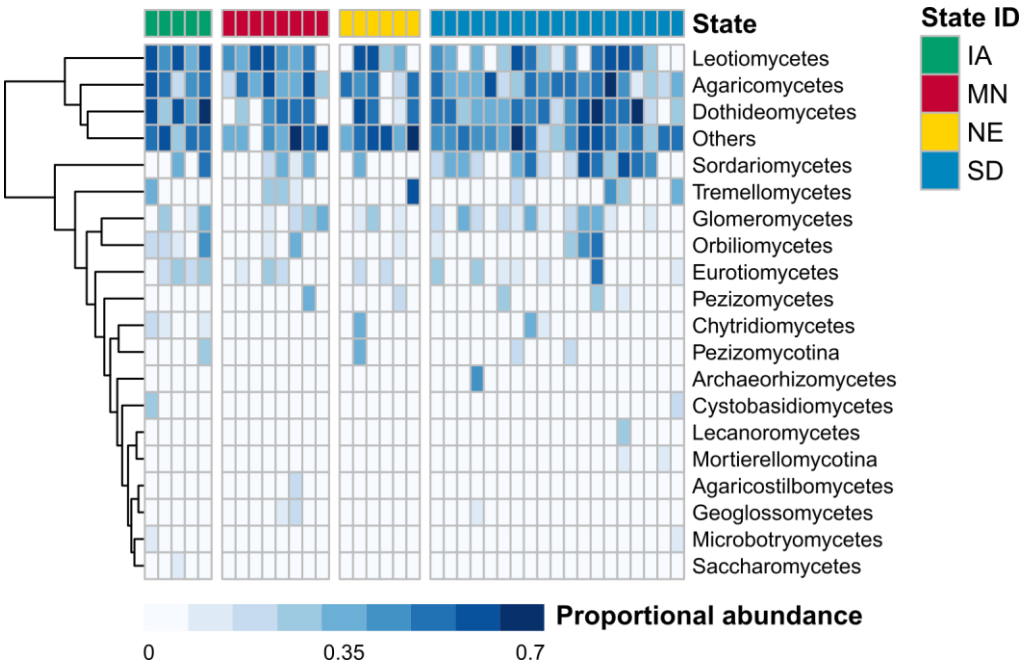

Figure 2.12. Abundance distributions of the top 25 percent eukaryotic classes in below-ground PCG tissue using the ITS1F/ITS4R primer set. Proportional abundances are displayed in a heatmap where color intensity correlates to abundance values. Rows indicate eukaryotic classes and columns denote individual PCG samples. Samples are partitioned by the state where they were collected from. State IDs signify either Iowa (IA), Minnesota (MN), Nebraska (NE), or South Dakota (SD). Rows are clustered via complete-linkage clustering. Class' abundance that is < 25% are partitioned into the category "Others".

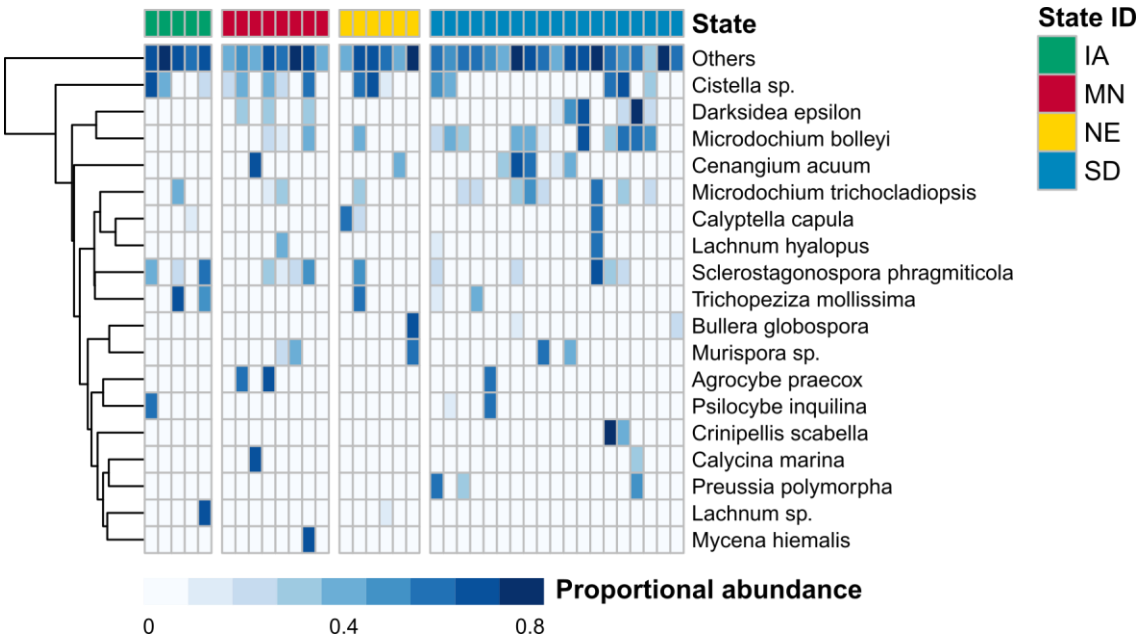

Figure 2.13. Abundance distributions of the top 25 percent eukaryotic species in below-ground PCG tissue using the ITS1F/ITS4R primer set. Proportional abundances are displayed in a heatmap where color intensity correlates to abundance values. Rows indicate eukaryotic phyla and columns denote individual PCG samples. Samples are partitioned by the state where they were collected from. State IDs signify either Iowa (IA), Minnesota (MN), Nebraska (NE), or South Dakota (SD). Rows are clustered via complete-linkage clustering. Species abundance that is < 25% is partitioned into the category "Others".

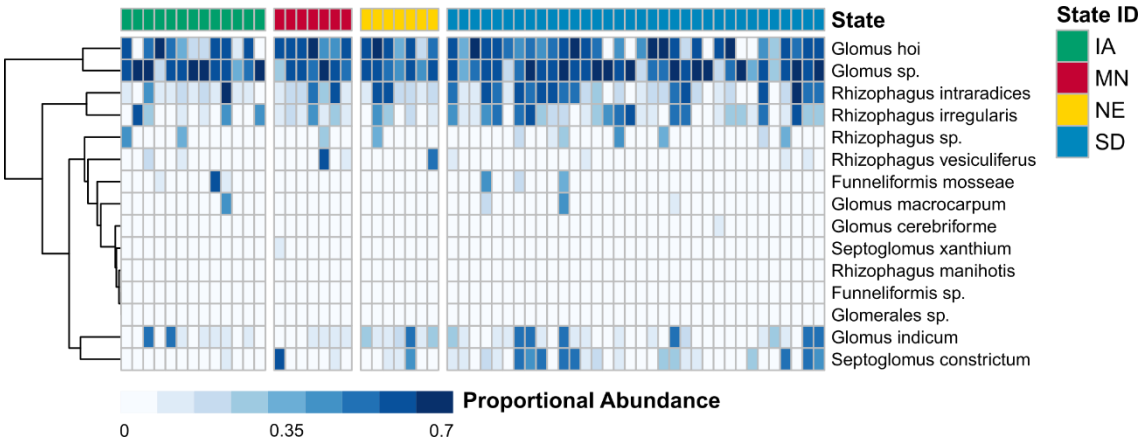

Figure 2.14. Abundance distributions of AM fungal species in below-ground PCG tissue using the AMV4.5NF/AMDGR primer set. Proportional abundances are displayed in a heatmap where color intensity correlates to abundance values. Rows indicate AM fungal species and columns denote individual PCG samples. Samples are partitioned by the state where they were collected from. State IDs signify either Iowa (IA), Minnesota (MN), Nebraska (NE), or South Dakota (SD). Rows are clustered via complete-linkage clustering.

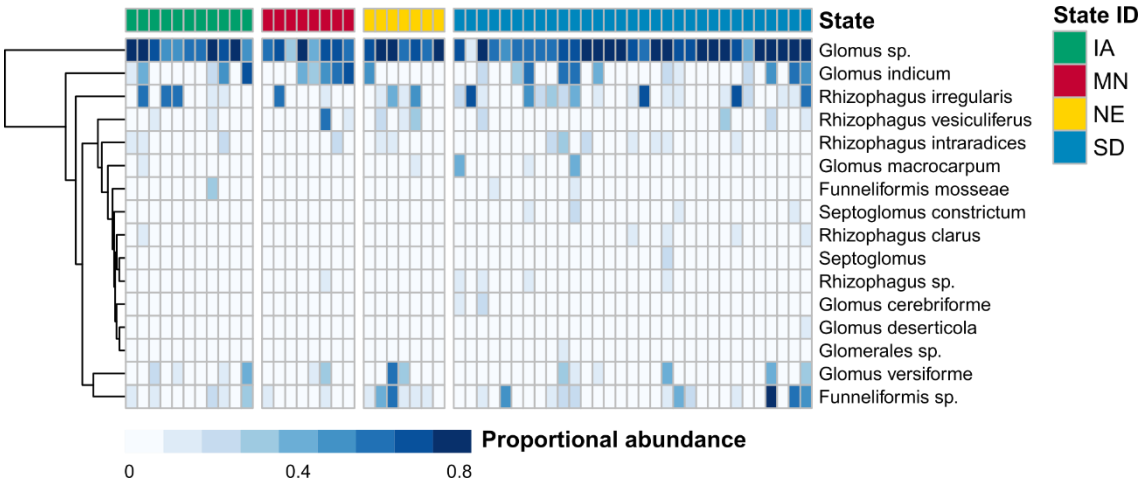

Figure 2.15. Abundance distributions of AM fungal species in below-ground PCG tissue using the NS31/AML2 primer set. Proportional abundances are displayed in a heatmap where color intensity correlates to abundance values. Rows indicate AM fungal species and columns denote individual PCG samples. Samples are partitioned by the state where they were collected from. State IDs signify either Iowa (IA), Minnesota (MN), Nebraska (NE), or South Dakota (SD). Rows are clustered via complete-linkage clustering.

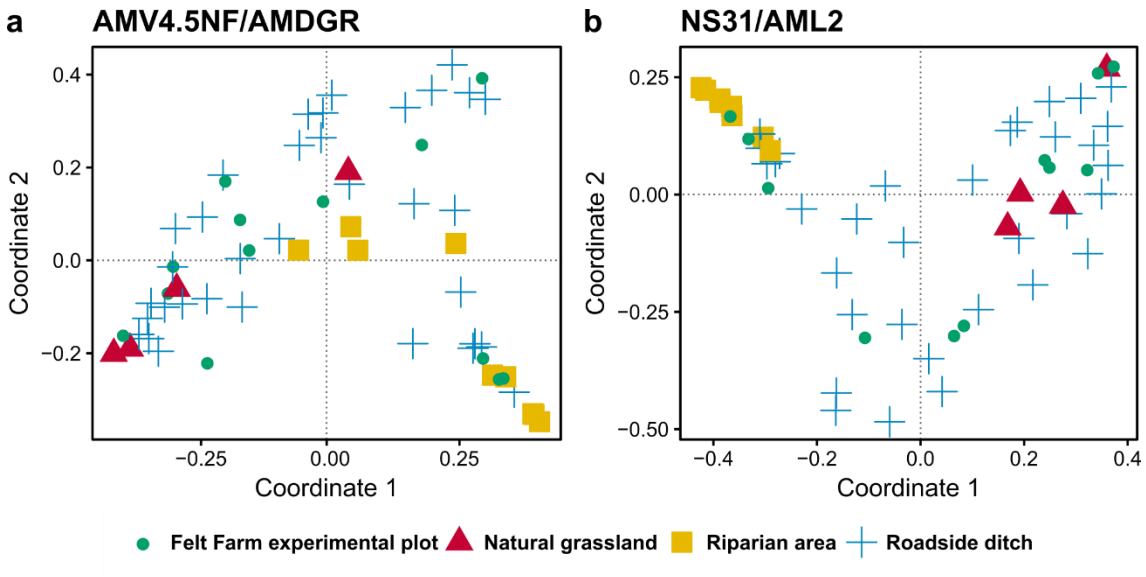

Figure 2.16. Principle coordinates analysis (PcoA) of AM fungal OTUs using Bray-Curtis dissimilarity metrics. PcoA biplots are based on Bray-Curtis distances amongst below-ground tissue of PCG. Data points are partitioned according to environmental location in which the PCG sample was collected via color and shape.

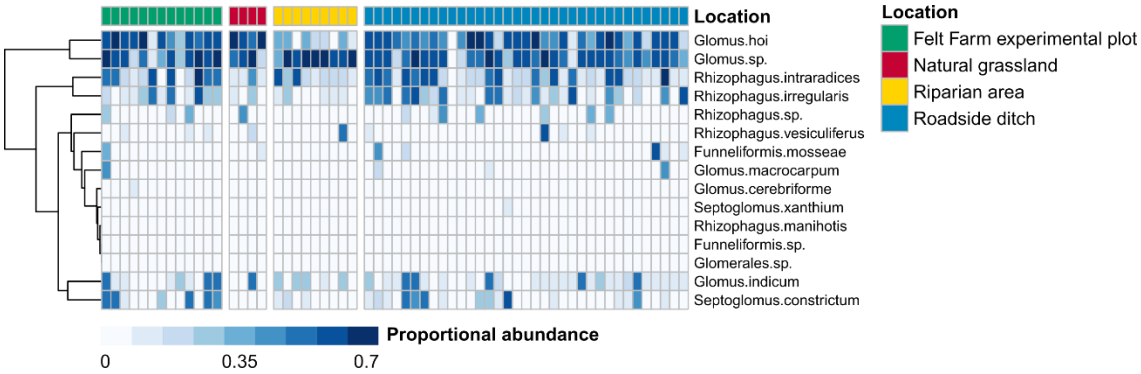

Figure 2.17. Abundance distributions of AM fungal species in below-ground PCG tissue using the AMV4.5NF/AMDGR primer set partitioned by environmental location. Proportional abundances are displayed in a heatmap where color intensity correlates to abundance values. Rows indicate AM fungal species and columns denote individual PCG samples. Samples are partitioned by the environmental location in which they were collected from. Location IDs signify either Felt Farm experimental plot (FF), natural grassland (NG), riparian area (RA), or roadside ditch (RD). Rows are clustered via complete-linkage clustering.

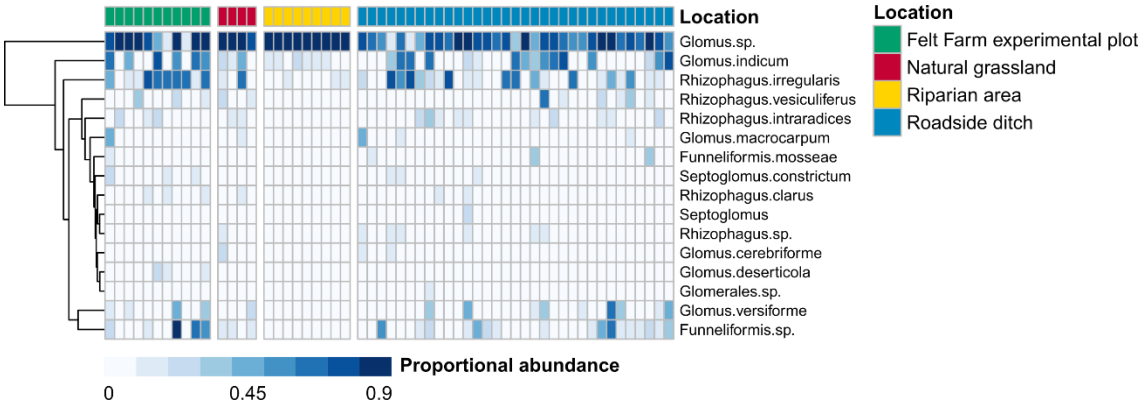

Figure 2.18. Abundance distributions of AM fungal species in below-ground PCG tissue using the NS31/AML2 primer set partitioned by environmental location. Proportional abundances are displayed in a heatmap where color intensity correlates to abundance values. Rows indicate AM fungal species and columns denote individual PCG samples. Samples are partitioned by the environmental location in which they were collected from. Location IDs signify either Felt Farm experimental plot (FF), natural grassland (NG), riparian area (RA), or roadside ditch (RD). Rows are clustered via complete-linkage clustering.

2.6 Tables

Table 2.1. Collection site locations for the microbiome analyses. Collection site locations for samples collected in Iowa (IA), Minnesota (MN), Nebraska (NE), and South Dakota (SD) in terms of latitude and longitude. Location column depicts one of the four environments in which the sample was found.

ID	State	Latitude	Longitude	Location
SP01	IA	43.2506	-95.3927	Natural grassland
SP02	IA	43.2545	-95.3607	Roadside ditch
SP03	IA	43.2452	-95.3947	Natural grassland
SP04	IA	43.2204	-95.4057	Natural grassland
SP05	IA	43.2139	-95.4557	Roadside ditch
SP06	IA	43.2312	-95.4057	Roadside ditch
SP07	IA	43.2526	-95.3946	Riparian area
SP08	IA	43.2425	-95.3949	Riparian area
SP09	IA	43.2045	-95.4117	Roadside ditch
SP10	IA	43.2040	-95.4058	Roadside ditch
SP11	IA	43.2011	-95.4058	Riparian area
SP12	IA	43.1912	-95.4102	Roadside ditch
SP13	IA	43.1860	-95.4047	Roadside ditch
SP14	MN	44.4563	-95.8569	Roadside ditch
SP15	MN	44.4558	-95.8569	Roadside ditch
SP16	MN	44.4920	-96.3431	Roadside ditch
SP17	MN	44.4789	-96.4040	Roadside ditch
SP18	MN	44.4489	-95.8569	Roadside ditch
SP19	MN	44.4824	-96.4042	Roadside ditch
SP20	MN	44.4951	-96.4041	Roadside ditch
SP21	MN	44.4494	-95.8568	Roadside ditch
SP22	NE	42.4647	-97.2910	Roadside ditch

Table 1 continued

ID	State	Latitude	Longitude	Location
SP23	NE	42.4713	-97.2702	Roadside ditch
SP24	NE	42.4714	-97.2660	Roadside ditch
SP25	NE	42.4712	-97.2537	Roadside ditch
SP26	NE	42.4712	-97.2514	Roadside ditch
SP27	NE	42.4650	-97.2923	Riparian area
SP28	NE	42.4714	-97.2534	Roadside ditch
SP29	NE	42.4650	-97.2914	Riparian area
SP30	SD	44.2307	-96.5867	Roadside ditch
SP31	SD	44.2303	-96.5712	Natural grassland
SP32	SD	44.2203	-96.5866	Riparian area
SP33	SD	44.2519	-96.9380	Natural grassland
SP34	SD	44.2539	-96.9373	Roadside ditch
SP35	SD	44.2539	-96.9365	Roadside ditch
SP36	SD	44.2535	-96.9994	Roadside ditch
SP37	SD	44.2536	-96.9983	Roadside ditch
SP38	SD	44.2537	-97.0330	Roadside ditch
SP39	SD	44.2536	-97.0319	Roadside ditch
SP40	SD	44.2537	-97.0312	Roadside ditch
SP41	SD	44.3690	-96.7951	Felt Farm experimental plot
SP42	SD	44.3684	-96.7957	Felt Farm experimental plot
SP43	SD	44.3678	-96.7976	Felt Farm experimental plot
SP44	SD	44.3566	-96.8873	Riparian area
SP45	SD	44.3557	-96.8873	Riparian area
SP46	SD	44.3553	-96.8868	Roadside ditch
SP47	SD	44.3553	-96.8853	Roadside ditch
SP48	SD	44.3559	-96.8875	Roadside ditch
SP49	SD	44.3564	-96.8876	Roadside ditch

Table 1 continued

ID	State	Latitude	Longitude	Location
SP50	SD	44.4253	-96.9693	Roadside ditch
SP51	SD	44.4257	-96.9699	Roadside ditch
SP52	SD	44.5289	-97.1290	Roadside ditch
SP53	SD	44.5287	-97.1282	Riparian area
SP54	SD	44.3678	-96.7976	Felt Farm experimental plot
SP55	SD	44.3678	-96.7975	Felt Farm experimental plot
SP56	SD	44.3678	-96.7976	Felt Farm experimental plot
SP57	SD	44.3678	-96.7975	Felt Farm experimental plot
SP58	SD	44.3678	-96.7975	Felt Farm experimental plot
SP59	SD	44.3690	-96.7952	Felt Farm experimental plot
SP60	SD	44.3690	-96.7951	Felt Farm experimental plot
SP61	SD	44.3690	-96.7951	Felt Farm experimental plot
SP62	SD	44.3690	-96.7952	Felt Farm experimental plot
SP63	SD	44.3691	-96.7951	Felt Farm experimental plot
SP64	SD	44.3679	-96.7961	Felt Farm experimental plot
SP65	SD	44.3679	-96.7959	Felt Farm experimental plot

Table 2.2. Primer information for amplicon generation. Five primer pairs were used for this experiment. Each pair consisted of two primers/sequences correlated to their specified target. Two primer pairs targeted taxa of arbuscular mycorrhizal fungi (AMF), while the other three targeted either nitrogen-fixing bacteria (diazotrophs), general prokarya, or general eukarya.

Target	**Amplicon Length**	**Primer**	**Sequence (5' – 3')**
AMF	300	AMV4.5NF	AAGCTCGTAGTTGAATTTCG
		AMDGR	CCCAACTATCCCTATTAATCAT
AMF	530	NS31	TTGGAGGGCAAGTCTGGTGCC
		AML2	GAACCCAAACACTTTGGTTTCC
Diazotrophs	360	F2	TGYGAYCCIAAIGCIGA
		R6	TCIGGIGARATGATGGC
Prokaryotic (general)	694	F357	TACGGGAGGCAGCAG
		R926	CCCCGTCAATTCCTTTGAGTTT
Eukaryotic (general)	> 600	ITS1F	TCCGTAGGTGAACCTGCGG
		ITS4R	TCCTCCGCTTATTGATATGC

Table 2.3. Demultiplexed run metrics. Total number of reads for both above- and below-ground tissue samples (***n***) are presented. Additionally, mean ($\bar{x}$) and standard deviation (***SD***) values are shown to display the spread of the data. Note: each read consists of an amplicon 300 nucleotides in length.

Tissue	***n***	**Total Reads**	$\bar{x}$	***SD***
Above-ground	65	5,549,924	51,550	12451.42
Below-ground	65	10,695,852	116,963	44485.86

Table 2.4. Primer sorted run metrics. This table shows the total number of reads for each primer pair in above- and below-ground prairie cordgrass tissue. Column ***n*** represents the number of samples that have reads ≥ 1. In addition to the total number of reads for each primer pair, mean ($\bar{x}$) and standard deviation (***SD***) values are shown to display the spread of the data. Note: each read consists of an amplicon 300 nucleotides in length.

Tissue	**Primer pair**	***n***	**Total Reads**	$\bar{x}$	***SD***
Above Ground	AMV4.5NF / AMDGR	63	6,258	48.891	17.64
	NS31 / AML2	62	754	5.891	3.11
	F2 / R6	13	3,542	27.672	205.08
	F357 / R926	65	5,530,856	43209.812	10422.72
	ITS1F / ITS4R	63	8,514	6.734	5.798
Below Ground	AMV4.5NF / AMDGR	65	6,026,064	47825.904	39469.707
	NS31 / AML2	58	528,226	4192.269	4220.818
	F2 / R6	3	6	0.049	0.2171
	F357 / R926	65	2,170,278	17502.241	12652.245
	ITS1F / ITS4R	59	1,692,210	13646.854	11435.422

Table 2.5. Read pair filtration metrics. Read pair (concatenation of reads one and two) counts pre- and post-filtration via *MOTHUR* quality control pipelines. Pair counts were analyzed for each tissue type and primer pair. Additionally, retention rate is reported as the number of reads kept after quality control filtration.

		Read pair metrics		
Tissue	**Primer pair**	**Pre-filtration**	**Post-filtration**	**% retention**
Above Ground	AMV4.5NF / AMDGR	3,129	0	0
	NS31 / AML2	377	0	0
	F2 / R6	1,771	0	0
	F357 / R926	2,765,428	1,289,299	46.62
	ITS1F / ITS4R	4,257	0	0
Below Ground	AMV4.5NF / AMDGR	3,013,032	1,956,578	64.94
	NS31 / AML2	264,113	143,124	54.19
	F2 / R6	3	0	0
	F357 / R926	1,089,805	515,405	47.29
	ITS1F / ITS4R	866,552	212,427	24.51

Table 2.6. Observed OTUs. Total number of OTUs for primer pairs that passed quality check steps in *MOTHUR*. OTUs are defined for sequences that share ≥ 97% similarity. Tissue type is denoted as either above-ground (AG) or below-ground (BG).

Primer pair	**Tissue**	**OTUs**
F357 / R926	AG	1161
F357 / R926	BG	4185
ITS1F / ITS1R	BG	597
AMV4.5NF / AMDGR	BG	141
NS31 / AML2	BG	100

Table 2.7. Alpha diversity metrics. Alpha diversity statistics for primer pairs that passed quality check steps in *MOTHUR*. Mean ($\bar{x}$), median ($\tilde{x}$), and standard deviation (***SD***) are presented for Shannon diveristy (Shannon) and Chao1 richness (Chao1) indexes. Tissue type is denoted as either above-ground (AG) or below-ground (BG).

		Shannon			**Chao1**		
Primer pair	**Tissue**	$\bar{x}$	$\tilde{x}$	**SD**	$\bar{x}$	$\tilde{x}$	**SD**
F357 / R926	AG	1.092	1.175	0.736	13.590	10.620	9.583
F357 / R926	BG	1.741	1.741	0.434	73.970	77.290	28.520
ITS1F / ITS1R	BG	1.010	1.006	0.464	11.350	10	6.554
AMV4.5NF / AMDGR	BG	0.738	0.792	0.424	5.749	6	1.916
NS31 / AML2	BG	0.341	0.231	0.327	5.412	5	3.748

Table 2.8. Analysis of variance results for Shannon diversity indexes. A two-way analysis of variance (ANOVA) was performed to determine variability of the Shannon diversity index in terms of the factors State or Location. ANOVA results are shown for each primer pair. Tissue type is denoted as either above-ground (AG) or below-ground (BG). "Significant" *p*-values are bolded with respective significance level.

Primer pair	**Tissue**	**Source**	**df**	**SS**	**MS**	**F**	***p*-value**
F357 /	AG	State	3	0.086	0.029	0.859	0.468
R926		Location	3	0.212	0.071	2.121	0.108
		Residuals	55	1.831	0.033		
F357 /	BG	State	3	7.494	2.498	3.5	**0.024***
R926		Location	3	10.206	3.402	4.767	**0.006****
		Residuals	39	27.832	0.714		
ITS1F /	BG	State	3	1.576	0.525	0.742	0.535
ITS4R		Location	2	0.569	0.284	0.401	0.673
		Residuals	32	22.669	0.708		
AMV4 /	BG	State	3	0.017	0.006	0.011	0.999
AMDGR		Location	3	1.802	0.601	1.109	0.354
		Residuals	54	29.259	0.542		
NS31 /	BG	State	3	0.132	0.044	0.174	0.913
AML2		Location	3	3.414	1.138	4.504	**0.007****
		Residuals	50	12.636	0.253		

Note: ***** $p < 0.1$; ****** $p < 0.05$; ******* $p < 0.001$

Table 2.9. Analysis of variance results for Chao1 diversity indexes. A two-way analysis of variance (ANOVA) was performed to determine variability of the Chao1 richness index in terms of the factors State or Location. ANOVA results are shown for each primer pair. Tissue type is denoted as either above-ground (AG) or below-ground (BG). "Significant" *p*-values are bolded with respective significance level.

Primer pair	**Tissue**	**Source**	**df**	**SS**	**MS**	**F**	***p*-value**
F357 /	AG	State	3	564.805	188.268	0.048	0.986
R926		Location	3	8304.141	2768.047	0.709	0.551
		Residuals	55	214783.619	3905.157		
F357 /	BG	State	3	374365.85	124788.617	3.275	**0.031****
R926		Location	3	270049.905	90016.635	2.363	**0.086***
		Residuals	39	1485984.499	38102.167		
ITS1F /	BG	State	3	1930.007	643.336	1.838	0.16
ITS4R		Location	2	163.656	81.828	0.234	0.793
		Residuals	32	11200.573	350.018		
AMV4 /	BG	State	3	362.454	120.818	0.858	0.469
AMDGR		Location	3	953.642	317.881	2.258	**0.092***
		Residuals	54	7603.707	140.809		
NS31 /	BG	State	3	956.083	318.694	0.711	0.55
AML2		Location	3	6936.952	2312.317	5.156	**0.003****
		Residuals	50	22424.036	448.481		

Note: ***** $p < 0.1$; ****** $p < 0.05$; ******* $p < 0.001$

Table 2.10. Permutational multivariate analysis of variance of Bray-Curtis dissimilarity indexes. PERMANOVA was performed to determine variability in terms of the Bray-Curtis dissimilarity index for either state or location. Bray-Curtis dissimilarities are based on abundance data for each primer pair. PERMANOVA results are shown for each primer pair. Tissue type is denoted as either above-ground (AG) or below-ground (BG). "Significant" *p*-values are bolded with respective significance level.

Primer pair	**Tissue**	**Source**	**df**	**SS**	**MS**	**Pseudo-*F***	R^2	***p*-value**
F357 /	AG	State	3	0.762	0.254	1.877	0.086	**0.084***
R926		Location	3	0.684	0.228	1.684	0.077	0.127
		Residuals	55	7.445	0.135		0.837	
F357 /	BG	State	3	0.41	0.137	0.902	0.053	0.56
R926		Location	3	1.354	0.451	2.98	0.176	**0.003****
		Residuals	39	5.909	0.152		0.77	
ITS1F /	BG	State	3	1.276	0.425	1.206	0.098	0.216
ITS4R		Location	2	0.507	0.254	0.72	0.039	0.769
		Residuals	32	11.280	0.352		0.863	
AMV4 /	BG	State	3	0.987	0.329	0.936	0.045	0.577
AMDGR		Location	3	1.887	0.629	1.789	0.087	**0.003****
		Residuals	54	17.925	0.351		0.825	
NS31 /	BG	State	3	1.099	0.366	1.011	0.05	0.434
AML2		Location	3	2.999	1	2.758	0.135	**< 0.001*****
		Residuals	50	17.034	0.362		0.768	

Note 1: ***** $p < 0.1$; ****** $p < 0.05$; ******* $p < 0.001$

Note 2: Each test was performed with 1,000 replicate permutations.

Table 2.11. PERMANOVA pairwise comparisons for Bray-Curtis distances based on state. All unique, pairwise combinations between each level of the location factor were analyzed for significant variability. Primer pairs that only showed statistical significance based on prior PERMANOVA testing were analyzed. Multiple-testing correction (*p*-value adj.) was performed using the false discovery rate (FDR). State levels are denoted as Iowa (IA), Minnesota (MN), Nebraska (NE), and South Dakota (SD). Tissue type is denoted as above-ground (AG).

Primer pair	**Tissue**	**Pairs**	**df**	**Pseudo-*F***	R^2	***p*-value**	***p*-value adj.**
F357 /	AG	SD vs MN	41	0.2	0.005	0.77	0.924
R926		SD vs IA	46	3.747	0.077	0.023	0.108
		SD vs NE	40	3.543	0.083	0.036	0.108
		MN vs IA	20	1.033	0.052	0.349	0.524
		MN vs NE	14	1.219	0.086	0.305	0.524
		IA vs NE	19	0.091	0.005	0.966	0.966

Table 2.12. PERMANOVA pairwise comparisons for Bray-Curtis distances based on location. All unique, pairwise combinations between each level of the location factor were analyzed for significant variability. Primer pairs that only showed statistical significance based on prior PERMANOVA testing were analyzed. Multiple-testing correction (*p*-value adj.) was performed using the false discovery rate (FDR). Location levels are denoted as roadside ditch (RD), natural grass land (NG), riparian area (RA), or Felt Farm experimental plot (FF). "Significant" adjusted *p*-values are bolded with respective significance level. Tissue type is denoted as below-ground (BG).

Primer pair	**Tissue**	**Pairs**	**df**	**Pseudo-*F***	**R2**	***p*-value**	***p*-value adj.**
F357 /	BG	RD vs NG	35	0.500	0.014	0.876	0.876
R926		RD vs FF	40	2.355	0.057	0.027	0.129
		RD vs RA	32	4.969	0.138	0.043	0.129
		NG vs FF	12	1.753	0.137	0.153	0.229
		NG vs RA	4	11.811	0.797	0.200	0.240
		FF vs RA	9	5.624	0.413	0.084	0.168
AMV4.5NF /	BG	RD vs RA	43	3.305	0.073	0.001	**0.006****
AMDGR		RD vs NG	38	0.931	0.025	0.481	0.668
		RD vs FF	47	0.797	0.017	0.668	0.668
		RA vs NG	12	2.384	0.178	0.013	**0.034****
		RA vs FF	21	2.100	0.095	0.017	**0.034****
		NG vs FF	16	0.856	0.054	0.611	0.668
NS31 /	BG	RD vs RA	41	5.472	0.12	0.001	**0.003****
AML2		RD vs NG	36	1.090	0.03	0.316	0.379
		RD vs FF	43	1.225	0.028	0.214	0.321
		RA vs NG	12	3.571	0.245	0.004	**0.008****
		RA vs FF	19	3.721	0.171	0.001	**0.003****
		NG vs FF	14	0.929	0.067	0.481	0.481

Note: ***** $p < 0.1$; ****** $p < 0.05$; ******* $p < 0.001$

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CHAPTER 3: IMPACT OF THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS ON THE BIOMASS POTENTIAL OF THE BIOENERGY CROP PRAIRIE CORDGRASS

3.1 Introduction

Spartina pectinata Link, also known as prairie cordgrass (PCG), is a warm season, perennial grass native to South Dakota ranging from sizes of 1 to more than 2 meters in height (Jensen 2006; Johnson et al. 2007) PCG has a broad distribution that spans throughout most the United States while also reaching all the way into the Arctic Circle of Northern Canada. C4-based photosynthesis makes prairie cordgrass more efficient at utilizing nitrogen, carbon dioxide, and water under limiting conditions (Alberts et al. 2002; Potter et al. 1995; Sage and Pearcy 1987), whilst decreasing the effects of photorespiration, which negatively affects C3-based plant systems.

PCG can grow in diverse soil conditions that include high levels of moisture (Skinner et al. 2009), well drained lands, and other stresses such as high levels of salinity (Anderson et al. 2015; Kim et al. 2011; Montemayor et al. 2008; Robben et al. 2018). From 2000 - 2008, populations of cordgrass produced on average 12.7 $Mg \cdot ha^{-1}$ of biomass (Boe et al. 2009) and were also able to outcompete switchgrass, another biofuel candidate, grown at the same locations (Boe and Lee 2007). Because of these unique abilities, it is crucial for further research to be conducted on this promising biofuel crop.

PCG can form symbiotic relationships with arbuscular mycorrhizal (AM) fungi, a member of the phylum, Glomeromycota. AM symbioses occurs in ca. 70 – 80% of all terrestrial plant species including economically important cereal crops (Smith et al. 2011) and potential feedstocks such as PCG (Liepold 2013). This symbiosis entails benefits for both organisms where AM fungi will obtain a fraction of the plant's carbon supplies and

in return, the plant will receive a plethora of benefits. One particular benefit involves the increased uptake of primarily phosphorus and some other nutrients including, nitrogen, sulfur, magnesium, copper, and zinc (Smith et al. 2011). Other beneficial traits include increased resistance to both biotic and abiotic stresses such as pathogenicity, drought, increased saline conditions, and heavy metal contamination of soils (Jeffries et al. 2003; Newsham et al. 1995; Singh et al. 2011). It has also been shown that these symbiotic relationships have increased carbon sequestration levels (Smith and Read 2008; Treseder and Holden 2013).

Despite our knowledge of these interactions amongst other plants and their significance, the level of research that has been performed on PCG has been rather limited. Previous research has shown that high rates of colonization can occur amongst PCG varieties native to this region of the United States and that there is the potential for increased levels of biomass development under limiting nutrient conditions. However, due to the high level of genetic variability found within PCG communities, it is still unclear if there is significant variation within this grass species in terms of biomass development, AM colonization rates, and nutrient acquisition. Therefore, our objectives of this research are as follows: (1) determine if genetic variability amongst PCG genotypes plays a role in biomass development, colonization rates, and nutrient efficiency; (2) Identify potential PCG genotypes that perform well under nutrient limiting conditions in terms of biomass production and mycorrhizal response.

3.2 Materials and Methods

3.2.1 Plant and fungal culture

We selected seven prairie cordgrass (PCG) genotypes with a high genetic variability based on a previous AFLP marker analysis of our local germplasm collection (Dwire 2010). Briefly, samples were collected from Minnesota, Iowa, North Dakota, South Dakota, Nebraska, and Kansas. In total, 152 samples from 87 different locations were used. Additionally, 10 samples from the "Red River" germplasm collection were analyzed. DNA was extracted from leaf tissue using methodology described by Doyle (1987). For AFLP analysis, the Applied Biosystems AFLP Plant Mapping Kit was used with modifications as described in Perkins et al. (2002). Selective amplification was performed using 6 combinations of primers. After selective amplification, analysis was performed on the amplicons using a Applied Biosystems ABI 3130XL Genetic Analyzer via electrophoresis. Electrophoretic peaks were visualized with Genescan v4.0 (Applied Biosystems, Foster City, California) between 50 and 500 bp. Any peak that was ≥ 75 relative fluorescent units (RFU) was classified as an allele. Peaks were scored as 1 or 0 for the presence or absence of alleles. Cluster analysis was performed using MVSP software (Kovach 1998). Genetic distance between restriction fragments was determined using the Nei and Li coefficient (Nei and Li 1979) in conjunction with the Cluster Analysis program in MVSP.

After the level of genetic variability was determined from our germplasm collection. Seven genotypes were selected depending on their location on the dendrogram produced via the cluster analysis. Genotypes located on branches that were spread throughout the dendrogram were picked due to higher proposed variability. Randomly

selected tillers from each of the seven genotypes were excised, washed thoroughly to remove all growth substrate, and transferred to sterile, tree seedling containers filled with 550 mL autoclaved growth substrate consisting of 70% sand, 20% organic top soil, and 10% perlite.

We used axenic mycorrhizal root organ cultures (ROC) of Ri T-DNA-transformed *Daucus carota* roots (clone DCI) to produce fungal inoculum. The mycorrhizal root systems were grown on mineral medium at 27° C (Bécard and Fortin 1988) for approximately 8 weeks. Sterile spores were isolated by blending the medium in 10 µM sodium citrate buffer at pH 6 for 1 minute. The solution was passed through a 22 µm filter paper, residual root material was manually removed, and the remaining spores were transferred into 250 mL of ultrapurified water.

3.2.2 Experimental design

In experiment 1, we examined genotypic variability in the mycorrhizal responsiveness of PCG under different nutrient supply conditions. After the PCG tillers were transplanted, we inoculated half of the plants with ca. 300 spores of the AM fungus *Rhizophagus irregularis* (DAOM 197198) (mycorrhizal) (Schüßler and Walker 2010) and the other half with filtration solution without spores (non-mycorrhizal). Each inoculation treatment was subjected to four modified Ingestad nutrient solutions supplied with the aforementioned nitrogen and phosphate amendments (-P/-N, -P/+N, +N/-P, or +N/+P) (Ingestad 1960). Phosphate and nitrogen concentrations were altered to simulate marginal soil conditions found in Eastern South Dakota based off of soil analysis from this area (Liepold 2013). The total nutrient profile is as follows: 1 mM NH_4NO_3, 0.05 mM KH_2PO_4, 0.617 mM KCl, 1 mM $CaCl_2 \cdot 2H_2O$, 0.015 mM Fe-

Ethylenediaminetetraacetic acid (EDTA), 0.625 mM $MgSO_4 \cdot 7H_2O$, 0.003 mM $MnCl_2 \cdot 4H2O$, 0.016 mM H_3BO_3, 0.113 μM Zn-EDTA, 0.372 μM $CuCl_2 \cdot 2H_2O$, and 0.034 μM $Na_2MoO_4 \cdot 2H_2O$. "+N" or "+P" signifies 1 mM or 0.05 mM of NH_4NO_3 or KH_2PO_4, respectively. "-N" or "-P" signifies 0 mM of either NH_4NO_3 or KH_2PO_4. These 4 modified Ingestad solutions were applied to plant systems every 3-4 weeks. Each nested treatment had 5 biological replicates and was organized in a randomized block design under greenhouse conditions (14-hour day, 10-hour night, 25°C, and watered every-other day with tap water). The plants were harvested after 332 days, and we measured the root and shoot biomass, AM root colonization, and the P and N contents of the plants.

To test whether the observed genotypic differences in the mycorrhizal responsiveness were dependent on the fungal species, we conducted a second experiment. In this experiment, we examined the effect of two different fungal isolates on three selected PCG genotypes under varying nutrient supply conditions. These three genotypes were selected on their responsiveness to the AM fungal inoculation in experiment 1 in terms of biomass development: a genotype that displayed strong positive responsiveness, a genotype that showed some positive responsiveness, and a genotype that showed no responsiveness. For this experiment, the plants were inoculated with ca. 300 spores of either *R. irregularis* (Blaszk., Wubet, Renker & Buscot; Walker & Schüßler, 2010; isolate 009 (M009) collected from southwest Spain by Mycovitro S.L. Biotechnología ecológica, Granada, Spain) or *Glomus aggregatum* (N.C. Schenck & G.S. Sm.; isolate 165 (M165) collected from the Long Term Mycorrhizal Research Site, University of Guelph, Guelph, Canada), or equal mixture of both fungal strains (MCOM). A non-mycorrhizal treatment like experiment 1 was also implemented. Each inoculation

treatment was subjected to three Ingestad nutrient solutions like experiment 1, however, the full nitrogen and phosphate amendment (+N/+P) was not used. Each nested treatment had 5 biological replicates which was organized in a randomized block design. The plants were grown for 348 days in the greenhouse as described above.

3.2.3 Biomass, responsiveness, and AM fungi colonization analyses

At the end of the growth period, roots and shoots were harvested, cleaned, and weighed for fresh biomass. New biomass growth was differentiated from the initial tiller weights for above ground tissue only. All below ground biomass was considered new due to preliminary size of the initial tiller. To determine PCG responsiveness to AM fungi, the following formula was used:

$$Responsiveness = \frac{Myc - NM}{NM}$$

where Myc is the mycorrhizal treatment and NM is the non-mycorrhizal treatment of a given variable. Values above zero were deemed to be a positive response (i.e. mycorrhizal treatments had higher values for a specific variable compared to the non-mycorrhizal control) and vice-versa. For the AM colonization analysis, an aliquot of the full root profile was excised, weighed, and fixed in 50 % ethanol for 72 h at room temperature. The rest of the root material and the shoots were dried at 60°C for 72 h and weighed for dry biomass. To determine total dry biomass of root material, water loss percentage from the remaining root material was applied to the fresh root aliquot weight excised for AM fungi colonization.

The fixed root material was washed with 10 % KOH (w/v) at 90°C for 1.5 h, rinsed three times with tap water and stained with 0.1 % (w/v) Chlorazol Black E in lacto-glycerol (lactic acid:glycerol:water; 13:12:16, v:v:v) for ca. 16 h at room

temperature. After the staining, the roots were transferred into lacto-glycerol and stored until further analysis. To quantify the AM fungal colonization rate, a modified grid-line intersect method was used (Giovannetti and Mosse 1980; McGonigle et al. 1990).

3.2.4 Phosphate and nitrogen analysis

For the P and N analysis, aliquots of roots and shoots were dried and ground using a Precellys 24 homogenizer (Bertin, Montigny-le-Bretonneux, France). We transferred approximately 20 mg of the ground tissue into 1.5 mL microcentrifuge tubes containing 1 mL of 2N HCl and incubated the samples at 95° C for 2 h. The samples were then vortexed, centrifuged at 12,000 · g for 5 m, and a 25 µL aliquot of the supernatant was added to 475 µL of type 1 water and 500 µL of ammonium molybdate vanadate (AMV), and the phosphate contents were quantified spectrophotometrically at 436 nm. Total nitrogen content was determined via dry combustion (900° C) using a Vario Max CN Elemental Analyzer (Elementar Analysensysteme, Langenselbold, Germany). Approximately 100 mg of dried and macerated above- and below-ground tissue was prepared and analyzed according to methodology described in Chintala et al. (2013).

3.2.5 Statistical analysis

Treatment effects were analyzed using a three-way analysis of variance (ANOVA) with PCG genotype, AM fungal inoculation, and nutrient regimen as the three dependent variables for both experiments. Paired t-tests were used to compare mycorrhizal and non-mycorrhizal treatments in experiment 1. Inoculation, nutrient, and genotypic variability was tested using Fisher's least significant (LSD) test (p-value ≤ 0.1). Correlations were analyzed using Pearson's correlation coefficient. All tests were conducted using the statistical software, R (R Core Team 2017).

3.3 Results

3.3.1 Biomass

In the first experiment, significant variability amongst the seven genotypes of prairie cordgrass (PCG) was observed under the differing nutrient input conditions (Table 3.1). While many of the genotypes analyzed varied depending on their respective fertilizer amendment, SP44.2 generally produced the lowest amount of biomass and SP67.1 produced the highest (Figure 3.1 and Figure 3.2). While the trend appears to be that the plants inoculated with AM fungi outperform the non-mycorrhizal plants, only a few genotypes are statistically significant. This is most notably observed under low input conditions (Figure 3.1). Under these conditions, the genotypes RR2, SP21.2, SP34.1 and SP67.1 developed the highest levels of above ground biomass under mycorrhizal conditions compared to their non-mycorrhizal counterparts and the other PCG genotypes. This trend is also observed in the below ground biomass. In general, below ground biomass took the highest partition of total biomass. The highest levels of below ground material were observed in SP44.2 once N amendments were implemented (Figure 3.3). While some significant differences were observed between fungal treatments, no definitive trend was identified in terms of biomass allocation (Table 3.2).

When analyzing the biomass data for the second experiment, significant variability ($p \leq 0.1$) was observed under low input conditions (Figure 3.4A) and when additional phosphorus was added to the substrate (Figure 3.4B). While no observable trend can be noticed from the fungal treatments, the non-mycorrhizal control (NM) generally produced the lowest amount of biomass when compared to its mycorrhizal counterparts. This can be observed in both Figure 3.4A and Figure 3.4C. The highest levels of biomass

production under low input conditions was from SP21.2 under combined mycorrhizal conditions. Under the same conditions, the lowest levels produced were from SP34.1 (Figure 3.4A). Similar results can be found when P was added to the substrate. While no significant variability amongst the fungal treatments in SP05.3 can be observed, SP21.2 and SP34.1 did show growth differences. The combined fungal treatment for SP21.2 once again, produced the highest levels of biomass for above and below ground material (Figure 3.4B). Additionally, significant variations in biomass can also be observed in PCG genotype comparisons (Table 3.3). Regardless of nutrient input, SP21.2 produced significantly higher biomass when compared to the other two genotypes (Figure 3.5).

Like experiment 1, many of the treatments observed displayed higher amounts of biomass in below ground material than in leaves and shoots. Significant variability could only be observed in SP21.2 between each of the fungal treatments. In this genotype, non-mycorrhizal plants had the highest levels of below ground material while both M009 and M165 treatments allocated more biomass to above ground material (Figure 3.6). While some variability was observed in SP21.2, no other variability was noticed between genotypes (Table 3.2).

We also observed the number of tillers and tiller weight. In both experiments, genotypic and nutrient variances were detected in terms of tiller count (Table 3.4) and biomass produced per tiller (Table 3.5). Variance in fungal treatments were also observed in both experiments. In experiment 1, SP21.2 displayed a positive response when treated AM fungi under low input conditions (Figure 3.7). SP34.1 also showed this trend when additional P was added to the substrate. In contrast, less tillers developed during the second experiment. While our data does display a trend of positive tiller development in

response fungal treatments, no real significant variability could be observed to support this prior claim (Figure 3.8).

3.3.2 Root colonization

All mycorrhizal plants had high levels of root colonization in both experiments. In the first experiment, significant variability ($p \leq 0.1$) was observed between the genotypes under the different nutrient input conditions (Table 3.6). Under low P and N amendments, SP22.1 had the lowest levels of colonization while the remaining genotypes had the highest levels and were statistically indistinguishable from one another (Figure 3.9A). More variability was observed when nitrogen was added to the soil substrate. RR2, SP21.2, and SP44.2 had the lowest colonization levels (Figure 3.9B). When phosphate was added to the substrate, SP34.1, SP44.2, and SP67.1 had the highest colonization levels (Figure 3.9C). When both phosphate and nitrogen were added to the soil substrate, SP44.2 had the highest levels of colonization (Figure 3.9D).

In the second experiment, no significant variation could be observed between only genotypes, but interactions between genotype and fungal treatment did show variance (Table 3.6). The lowest recorded colonization rates were identified under high P conditions (Figure 3.10C). These were determined to be from SP05.3 and SP21.2 for M165 and M009, respectively. Conversely, the highest colonization rates were in SP21.2 when treated with M165 under low input conditions (Figure 3.10A). To see how well root colonization related to total P content and biomass of the plant, linear regression was performed. For both experiments, root colonization had a low correlation coefficient when compared to total P content and biomass (Figure 3.11).

3.3.3 P and N nutrition

In the first experiment, higher levels of variability between fungal treatments were found within the phosphate content when compared to the nitrogen content, most notably in the above ground biomass (Table 3.8, and Table 3.7). While nitrogen content displayed minimal changes in fungal treatments above ground, higher levels of significant variability were observed in below ground tissue. These treatments generally showed that non-mycorrhizal plants acquired more N than their mycorrhizal counterpart. This was predominantly observed under low input conditions where nitrogen was limited (Figure 3.12).

Subsequently, the genotypes SP21.2 and SP67.1 generally garnered the highest levels of phosphate, while SP44.2 accumulated the least amongst many of the input conditions observed. One exception to this trend would be in the non-mycorrhizal condition of SP44.2 under low input conditions which contained the second highest mean and most significant value of phosphorus. The highest levels of phosphoric variability can be observed under low P and N in above ground tissue. Under mycorrhizal conditions, the phosphate levels were significantly higher ($p \leq 0.1$) in SP21.2, RR2, and SP67.1 in either above or below ground tissue. The lowest levels of phosphate were generally observed in the non-mycorrhizal conditions (Figure 3.13). This variability tends to diminish once phosphate or nitrogen is added to the soil substrate (Figure 3.13B). One exception to this observation is found in SP21.2 under mycorrhizal conditions with only additional phosphate added to the soil mixture (Figure 3.13C). When both phosphate and nitrogen are added to the soil substrate, variability is also diminished between mycorrhizal and non-mycorrhizal conditions, specifically in above ground biomass but

varied between the PCG genotypes. The below ground biomass followed this similar trend, however, the mycorrhizal condition of SP67.1 was significantly higher compared to its non-mycorrhizal counterpart (Figure 3.13D).

In the second experiment, only phosphate content was analyzed since the nitrogen data in the prior trial showed low levels of genotypic variability and broad value ranges within treatments (Figure 3.14) when compared to phosphate values (Figure 3.15). PCG genotype, fungal treatment and interactions between these two showed significant variability both above- and below-ground biomass (Table 3.9). The highest levels of total P were identified under low input conditions and when additional phosphate was added to the substrate. Under low input conditions, the highest levels of phosphate were found in SP21.2 with the combined fungal treatment and in SP34.1 when inoculated with *G. irregularis* 09 (M009) (Figure 3.16A). When phosphate was added to the substrate, variability increased mainly in below ground plant material. The highest observed amounts of P can be identified in SP21.2 under combined fungal conditions which contained an almost identical amount to the same genotype under low input conditions (Figure 3.16C). Like the biomass development, phosphate content mirrored these prior trends. SP21.2 contained the highest levels of phosphate while SP34.1 contained the lowest (Figure 3.17).

To see how well the total P content related to the overall biomass of the plant, simple linear regression was performed in both experiments. The results from this analysis revealed a strong correlation between these two variables. Many of the data points tend to localize around the origin of the plot while the more extreme values were identified to be from the genotype SP21.2 and SP67.1 (Figure 3.18).

3.4 Discussion

This study has shown the effect of AM fungi inoculation on growth of PCG under varying nutrient conditions. Our results suggest that genotypic variability of PCG affects mycorrhizal responsiveness in terms of biomass development. In experiment 1, Four out of the seven PCG genotypes inoculated with R. irregularis DAOM197198 had significantly higher levels of biomass compared to their non-mycorrhizal counterparts when neither N or P is supplied to the substrate. Once N or P is added to the substrate, growth responses are diminished. In experiment 2, similar results were also observed with *G. intraradices* 09 and *G. aggregatum* 165. The results from experiment 2 show that differing AM fungal species can independently affect biomass production.

This mycorrhizal growth response trend has also been described by others (Menge et al. 1978; Mosse 1973; Smith et al. 1986). It has also been shown that plants receive more growth promoting benefits from AM symbiosis in nutrient limited soils compared to nutrient rich soils (Koorem et al. 2014; Teste et al. 2014). Once N or P was added to the to the growth substrate, negative mycorrhizal growth responses were observed in two genotypes. This response can be the result of nutritional benefits received from AM fungi being less than the carbon costs from the plant. This concept has been generally accepted as the cause of negative mycorrhizal growth responses (Johnson et al. 1997; Smith and Smith 2013). Despite these two exceptions, most the genotypes under high nutrient conditions showed no difference between NM and AM fungi inoculated treatments.

Furthermore, our biomass data show that genotypic variability of PCG also affects biomass development under various nutrient input conditions. Similar differences in biomass production have been observed in populations of PCG collected from South

Dakota (Boe and Lee 2007; Boe et al. 2009). This is also observed in cultivars and populations of switchgrass, another potential bioenergy crop (Casler 2005; Casler and Boe 2003; Das et al. 2004).

In experiment 2, co-inoculation of two AM fungal species with PCG showed high mycorrhizal growth responses compared to non-mycorrhizal and single inoculation treatments. This result indicates that fungal competition has the capacity to improve mycorrhizal growth responses. Hepper et al. (1988) describe dual inoculum strategies implicating an increased fresh weight response with multiple combinations of AM fungi. Werner and Kiers (2015) also displayed this phenomenon. In this study, native species were invaded with subsequent AM fungi. The overall trend indicated that dry biomass values were higher when more than one AM fungi was introduced to the plant as opposed to when only one AM fungi was applied to the host plant. Thonar et al. (2014) explained this as well. AM fungi inoculant combinations outperformed their singular counterparts. This effect can be explained by additive benefits given by both AM fungal species to the plant. Since PCG is the sole source of C assimilates in these experiments, the plant can discriminate against AM fungi that are less cooperative. This discrimination can force AM fungi to become more cooperative therefore leading to additive effects. This concept has been elucidated by (Kiers et al. 2011).

Our studies show that AM fungi are highly associated with PCG. This is consistent with the findings by Dhillion and Friese (1994). In this study, they identified a plethora of plant species found within the prairies of North America that were colonized by AM fungi. Particularly, PCG was found to be highly colonized. Anderson et al. (1986) also found that PCG can be colonized under variable soil moisture content.

Our findings also show that these high colonization rates do not always reflect higher nutrient exchange or increased biomass. Anderson et al. (1986) showed that the relationship between root colonization in PCG and fresh tiller biomass is not positively correlated. Other research has shown varying effects of AM fungal colonization on biomass and nutrient uptake. A meta-analysis conducted by Treseder (2013) revealed that when root colonization increases, plant growth and P nutrition will frequently increase as well. However, the data presented showed high levels of variability amongst the studies used in the analysis. This variability was attributed to differences in how data was obtained. Another meta-analysis conducted by Lekberg and Koide (2005) showed similar results however a significant portion of the studies analyzed show no or reduced effects on biomass in terms of colonization. As some of these studies have pointed out, there are some pitfalls to this percent colonization technique. First, the measurement only takes a "snapshot" of colonization at one temporal point. Secondly, these techniques account for all fungal structures present inside the root cortex; they do not account for what components are active and inactive.

Our findings from both experiments suggest that biomass increase is the result of increased total phosphate (P) to the plant. This indicates that for PCG to gain more biomass, an increased uptake of P is needed. Positive growth impacts observed by the AM symbiosis can be attributed to improved P nutrition. Comprehensive research has been performed to substantiate this claim (Smith et al. 2003). Smith et al. (2009) showed evidence that AM fungi can even provide the main sources of P for plants, regardless of growth responses of the plant.

In contrast, the results from experiment one indicates that biomass increase is not the result of increased nitrogen (N) to the plant. Additionally, very little significant difference in N content was observed between the mycorrhizal and non-mycorrhizal treatments for above ground biomass. This was also prevalent amongst the high and low nutrient regimens. The most variability was observed under low input conditions for below ground material. Like P, N uptake has also been shown to be increased by AM symbioses, albeit in limited studies (Cavagnaro et al. 2006; George et al. 1995). However, it remains unclear how this process fully works (Atul-Nayyar et al. 2009). On the contrary, other studies have shown that AM symbioses do not effect N nutrition (Ames et al. 1983; Hawkins et al. 2000; Reynolds et al. 2005). Due to PCG's growth not being limited to N, our data suggests that P is the growth limiting factor in development for this experiment (Agren et al. 2012).

3.5 Figures

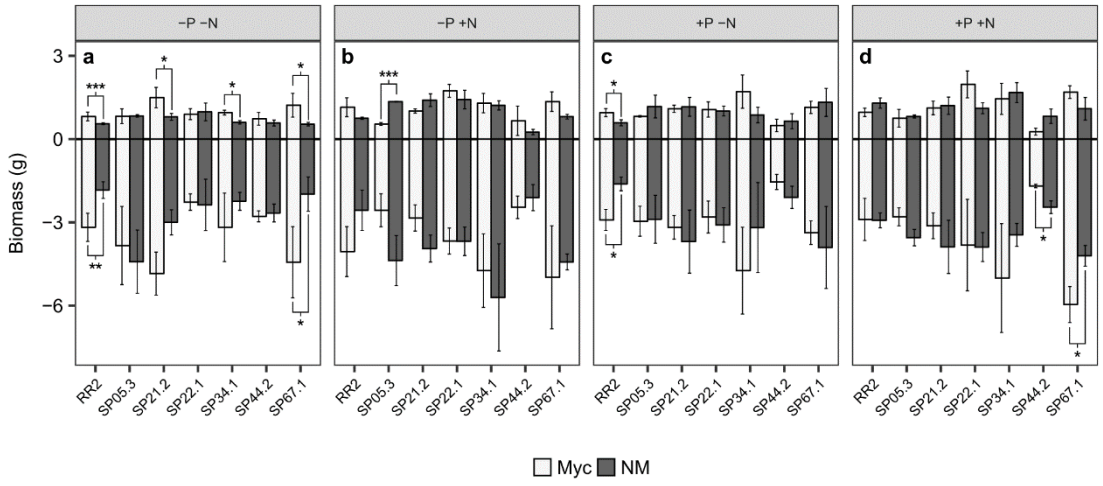

Figure 3.1. Experiment 1: mean biomass value comparisons by fungal treatment. Mean dry biomass values are presented in terms of both above ground (bars ascending from zero) and below ground (bars descending from zero) values. Error bars represent the standard error of the mean (SEM). Values are partitioned according to nutrient input condition: low P and low N (A), low P and high N (B), high P and low N (C), high P and high N (D). The lighter shaded bars reflect plants inoculated with mycorrhizae (Myc) while darker shaded bars represent plants not inoculated with mycorrhizae (NM). Asterisks indicate results of independent, t-test comparisons between the mycorrhizal treatment and non-mycorrhizal control; *p*-value ≤ 0.1 (*), *p*-value ≤ 0.05 (**), *p*-value ≤ 0.01 (***).

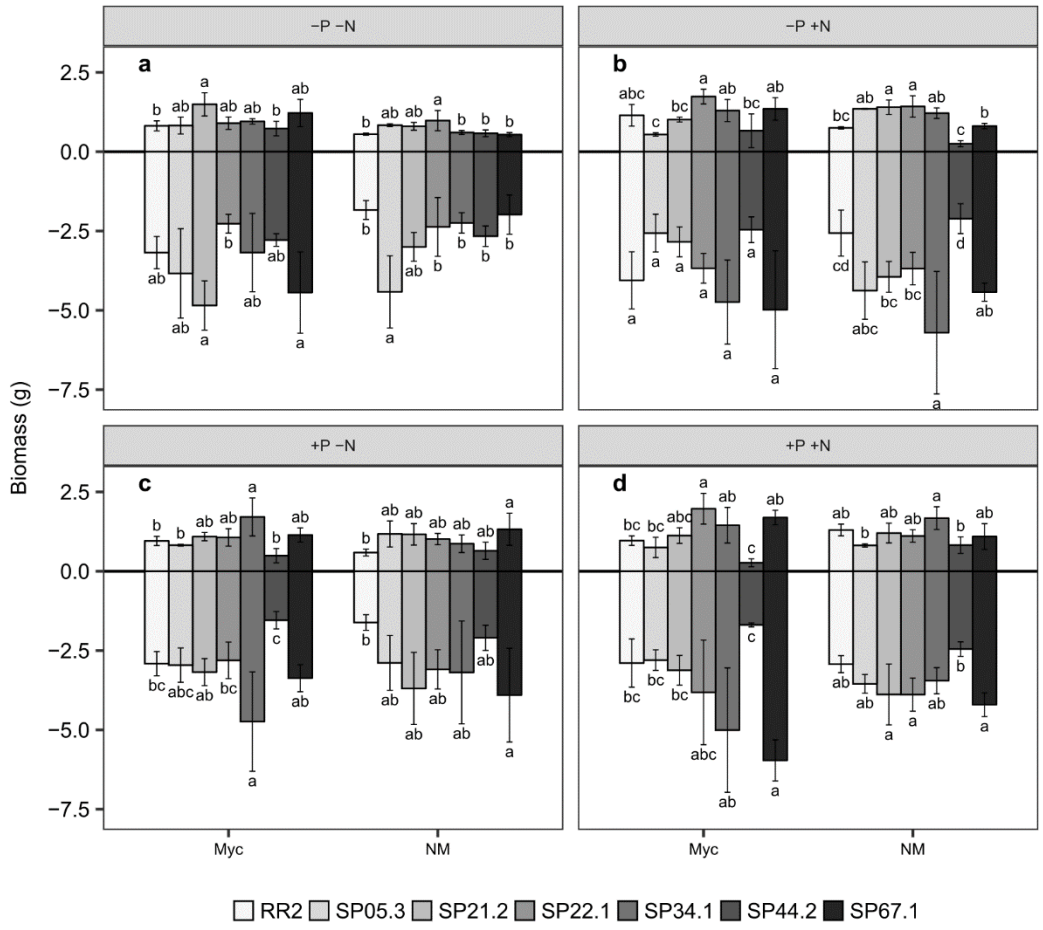

Figure 3.2. Experiment 1: mean biomass value comparisons by PCG genotype. Mean dry biomass values are presented in terms of both above ground (bars ascending from zero) and below ground (bars descending from zero) values. Error bars represent the standard error of the mean (SEM). Values are partitioned according to nutrient input condition: low P and low N (A), low P and high N (B), high P and low N (C), high P and high N (D). Within each partition, values are separated by fungal treatment (Myc) or non-mycorrhizal control (NM). Bar colors denote PCG genotype. Letters above and below each bar denote the results of the Fisher's least significant difference (LSD) test (p-value ≤ 0.1). LSD comparisons were made between each PCG genotype for each AM fungal treatment in nutrient partitions.

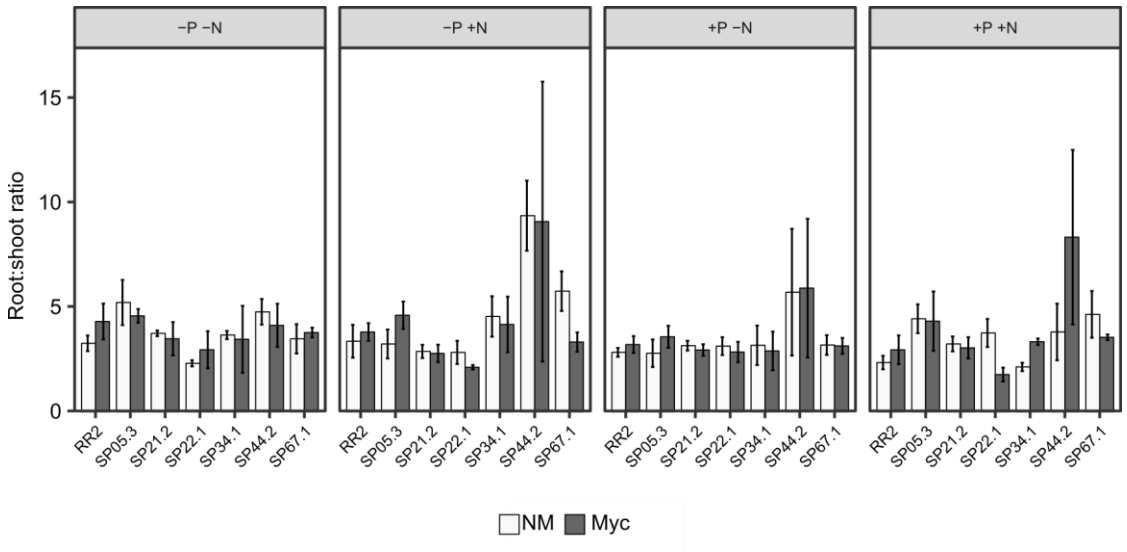

Figure 3.3. Experiment 1: mean root:shoot ratio comparisons by fungal treatment. Bar height represents mean root:shoot ratios for experiment 1. Error bars represent the standard error of the mean (SEM). Values are partitioned according to nutrient input condition: low P and low N (A), low P and high N (B), high P and low N (C), high P and high N (D). The lighter shaded bars reflect plants inoculated with mycorrhizae (Myc) while darker shaded bars represent plants not inoculated with mycorrhizae (NM). No significant variation was observed between Myc and NM treatments.

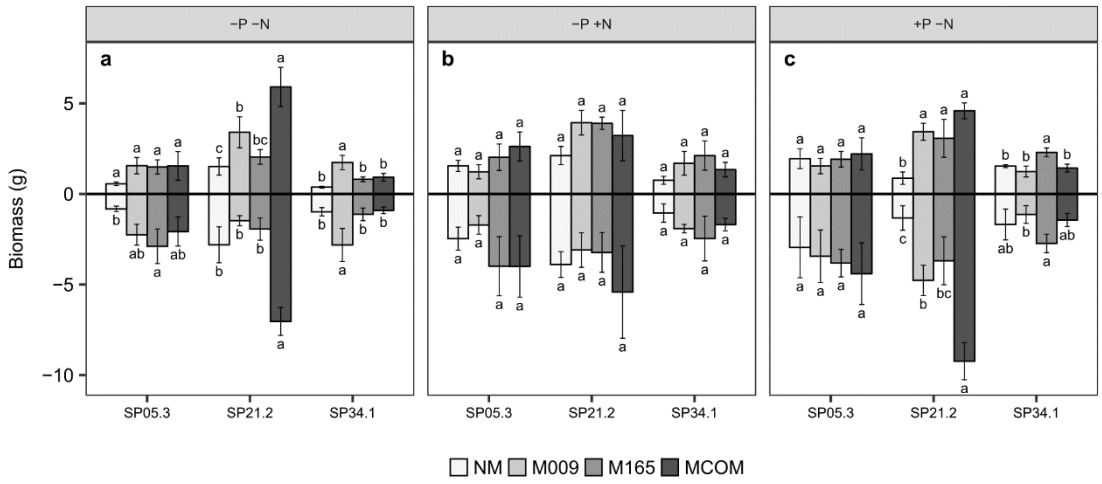

Figure 3.4. Experiment 2: mean biomass value comparisons by fungal treatment. Mean dry biomass values are presented in terms of both above ground (bars ascending from zero) and below ground (bars descending from zero) values. Error bars represent the standard error of the mean (SEM). Values are partitioned according to nutrient input conditions: low P, low N (A), low P and high N (B) and high P and low N (C). White bars indicate non-mycorrhizal plant treatments, light gray indicates *R. irregularis* isolate 009, medium gray indicates *G. aggregatum* isolate 165, and dark gray bars signify an equal combination of both 009 and 165 AM fungal isolates. Letters above and below each bar denote the results of the Fisher's least significant difference (LSD) test (p-value ≤ 0.1). LSD comparisons were made between each fungal treatment for each PCG genotype.

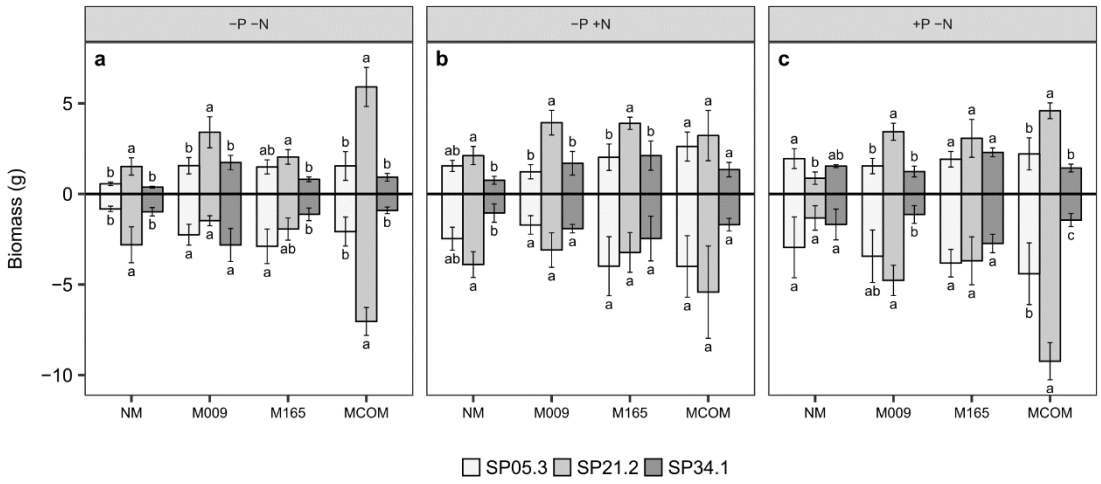

Figure 3.5. Experiment 2: mean biomass value comparisons by PCG genotype. Mean dry biomass values are presented in terms of both above ground (bars ascending from zero) and below ground (bars descending from zero) values. Error bars represent the standard error of the mean (SEM). Values are partitioned according to nutrient input conditions: low P, low N (A), low P and high N (B) and high P and low N (C). Bar colors signify PCG genotype. Letters above and below each bar denote the results of the Fisher's least significant difference (LSD) test (p-value $\leq$ 0.1). LSD comparisons were made between each PCG genotype for each AM fungal treatment.

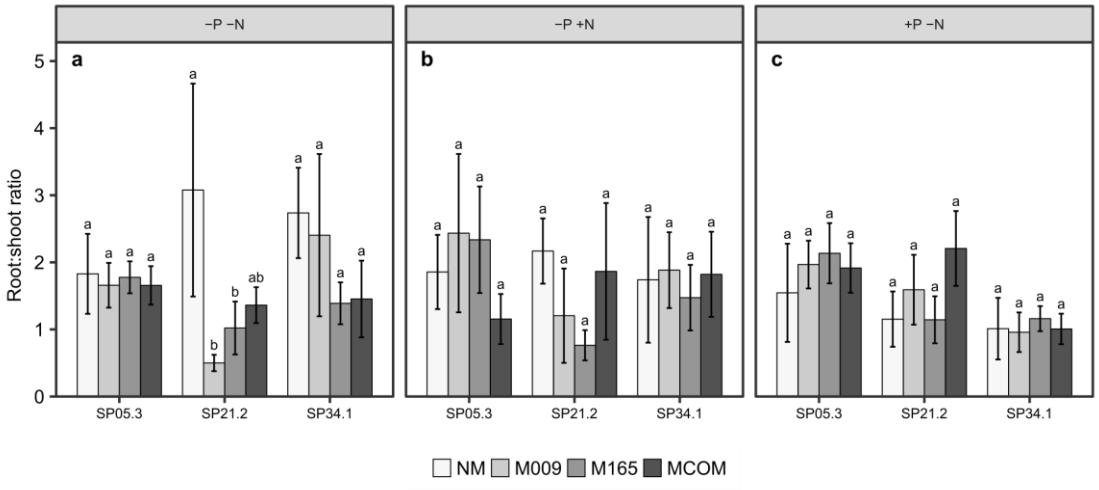

Figure 3.6. Experiment 2: mean root:shoot ratio comparisons by fungal treatment. Bar height represents mean root:shoot ratios for experiment 2. Error bars represent the standard error of the mean (SEM). Values are partitioned according to nutrient input conditions: low P, low N (A), low P and high N (B) and high P and low N (C). White bars indicate non-mycorrhizal plant treatments, light gray indicates *R. irregularis* isolate 009, medium gray indicates *G. aggregatum* isolate 165, and dark gray bars signify an equal combination of both 009 and 165 AM fungal isolates. Letters above each bar denote the results of the Fisher's least significant difference (LSD) test (p-value ≤ 0.1). LSD comparisons were made between each fungal treatment for each PCG genotype.

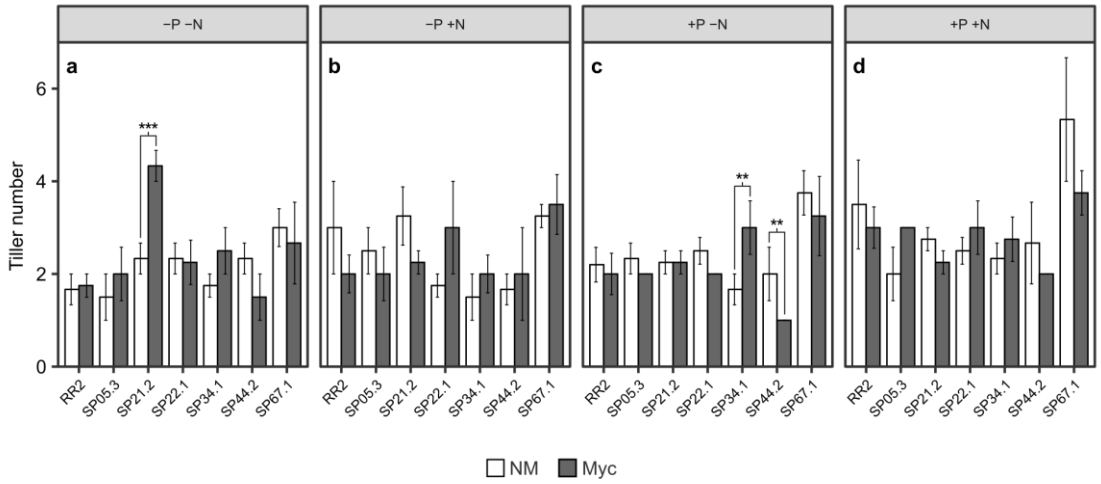

Figure 3.7. Experiment 1: mean tiller number comparisons by fungal treatment. Bar height represents tiller numbers for experiment 1. Error bars represent the standard error of the mean (SEM). Values are partitioned according to nutrient input condition: low P and low N (A), low P and high N (B), high P and low N (C), high P and high N (D). The lighter shaded bars reflect plants inoculated with mycorrhizae (Myc) while darker shaded bars represent plants not inoculated with mycorrhizae (NM). Asterisks indicate results of independent, t-test comparisons between the mycorrhizal treatment and non-mycorrhizal control; p-value ≤ 0.1 (*), p-value ≤ 0.05 (**), p-value ≤ 0.01 (***).

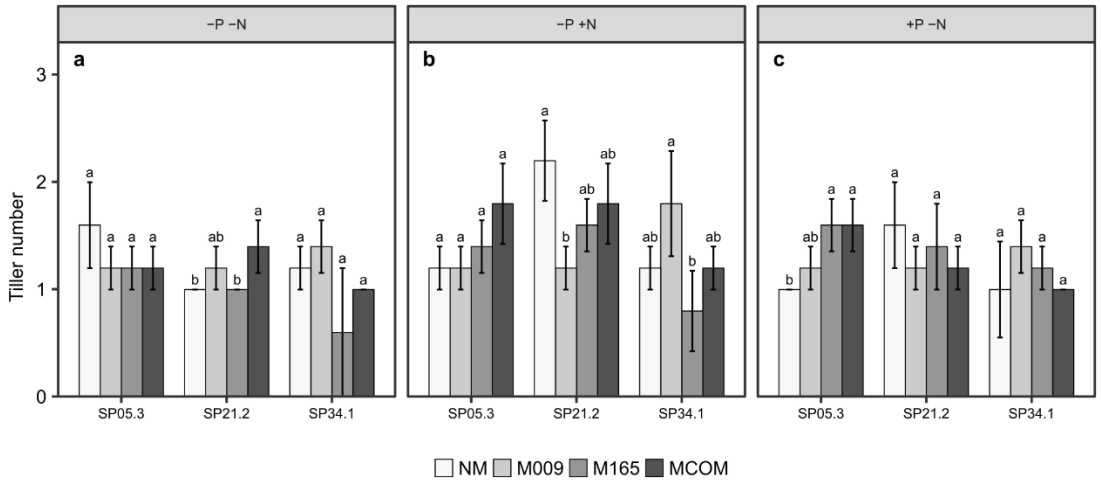

Figure 3.8. Experiment 2: mean tiller number comparisons by fungal treatment. Bar height represents mean tiller numbers for experiment 2. Error bars represent the standard error of the mean (SEM). Values are partitioned according to nutrient input conditions: low P, low N (A), low P and high N (B) and high P and low N (C). White bars indicate non-mycorrhizal plant treatments, light gray indicates *R. irregularis* isolate 009, medium gray indicates *G. aggregatum* isolate 165, and dark gray bars signify an equal combination of both 009 and 165 AM fungal isolates. Letters above each bar denote the results of the Fisher's least significant difference (LSD) test (p-value ≤ 0.1). LSD comparisons were made between each fungal treatment for each PCG genotype.

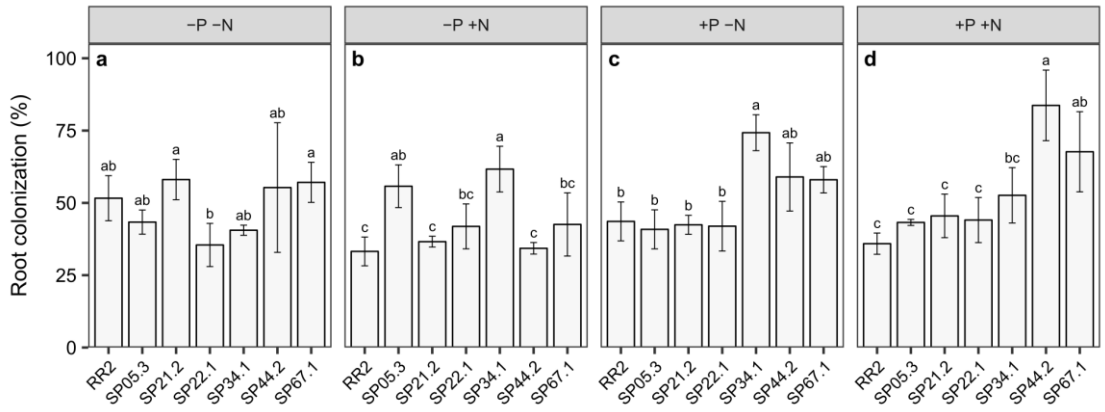

Figure 3.9. Experiment 1: mean root colonization comparisons by genotype. Mean root colonization percentages are visualized in terms of bar height. Error bars represent the standard error of the mean (SEM). Values are partitioned according to nutrient input condition: low P and low N (A), low P and high N (B), high P and low N (C), high P and high N (D). Letters above each bar denote the results of the Fisher's least significant difference (LSD) test (p-value ≤ 0.1). LSD comparisons were made between each PCG genotype for each nutrient partition.

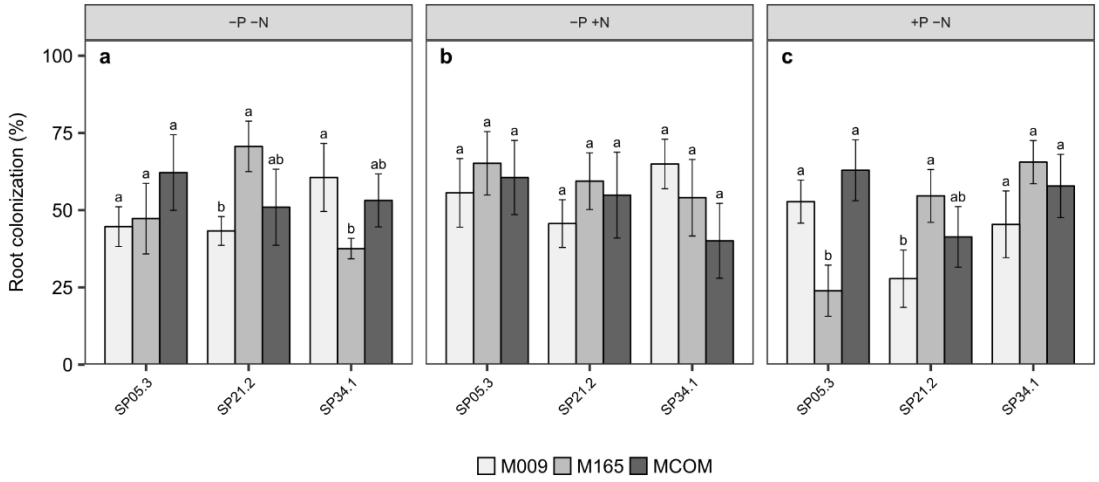

Figure 3.10. Experiment 2: mean root colonization comparisons by genotype. Mean root colonization percentages are visualized in terms of bar height. Error bars represent the standard error of the mean (SEM). Values are partitioned according to nutrient input condition: low P and low N (A), low P and high N (B), high P and low N (C). Light gray bars represent *R. irregularis* isolate 009, medium gray indicates *G. aggregatum* isolate 165, and dark gray bars signify an equal combination of both 009 and 165 AM fungal isolates. Letters above each bar denote the results of the Fisher's least significant difference (LSD) test (*p*-value ≤ 0.1). LSD comparisons were made between each PCG genotype for each nutrient partition.

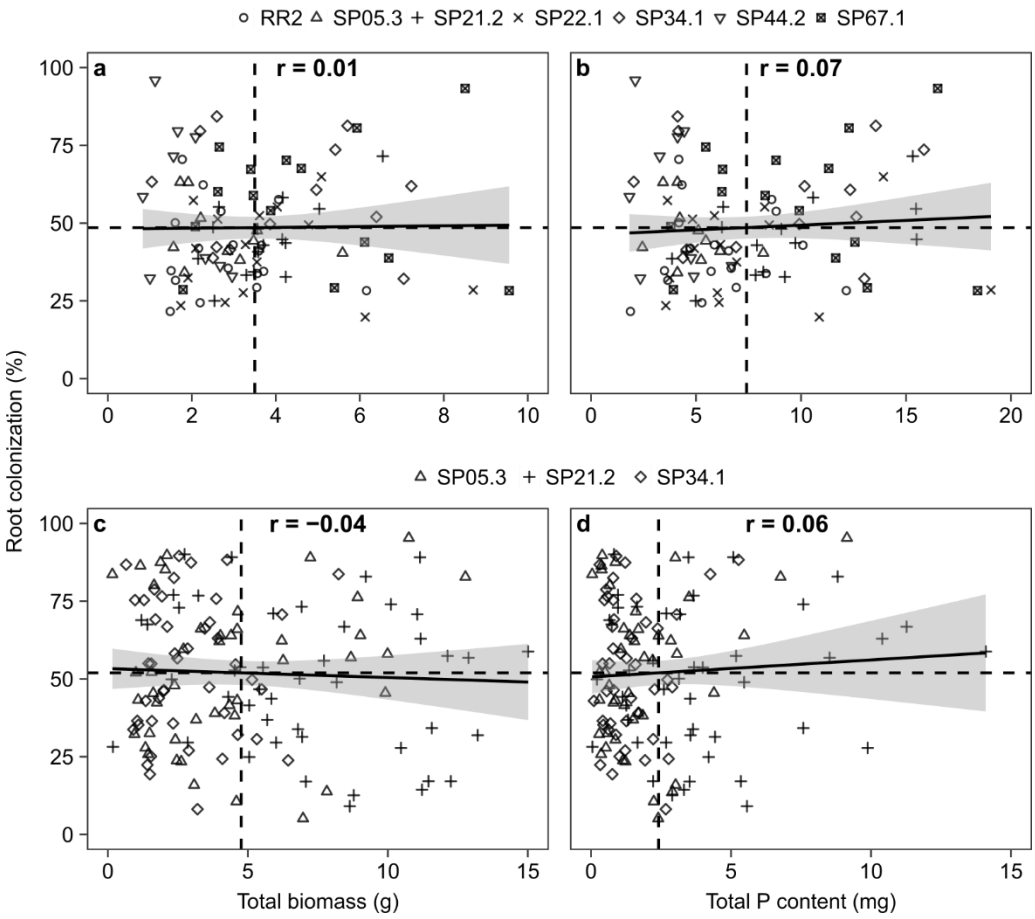

Figure 3.11. Experiments 1 & 2: linear regression of root colonization vs. biomass and P content. Comparisons between the variables total biomass and root colonization (A and C) and total P content and root colonization (B and D) were made for all mycorrhizal treatments in experiment 1 (A and B) and experiment 2 (C and D). Plot nodes are distinguished by their respective genotype (shape). Shaded regions signify the 95% confidence interval of the regression slope. Dotted lines represent mean values for both x and y variables.

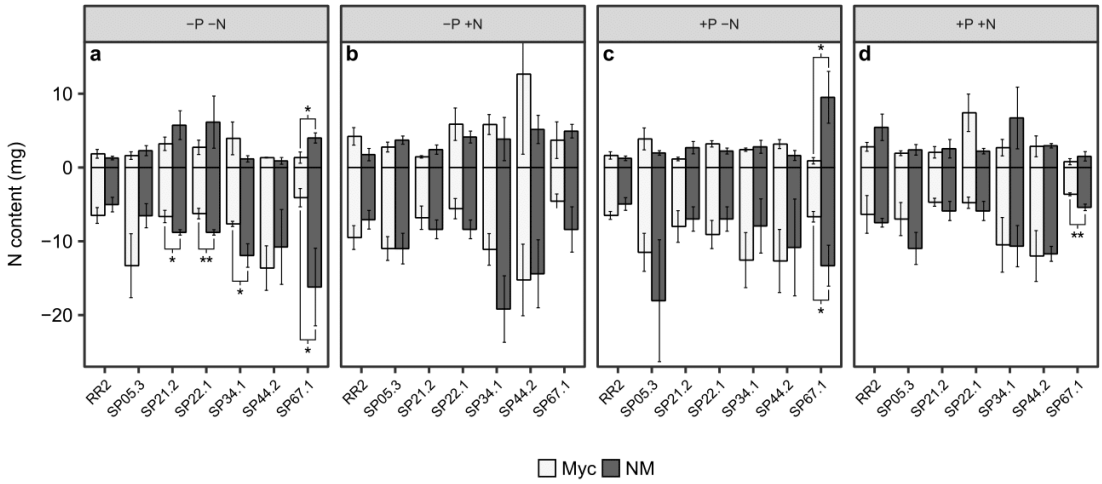

Figure 3.12. Experiment 1: mean N content value comparisons by fungal treatment. Mean N content values are presented in terms of both above ground (bars ascending from zero) and below ground (bars descending from zero) tissue values. Error bars represent the standard error of the mean (SEM). Values are partitioned according to nutrient input condition: low P and low N (A), low P and high N (B), high P and low N (C), high P and high N (D). The lighter shaded bars reflect plants inoculated with mycorrhizae (Myc) while darker shaded bars represent plants not inoculated with mycorrhizae (NM). Asterisks indicate results of independent, t-test comparisons between the mycorrhizal treatment and non-mycorrhizal control; p-value ≤ 0.1 (*), p-value ≤ 0.05 (**). Note: the mycorrhizal treatment error bar of genotype SP44.2 in partition B has been cropped out viewing area to increase resolution of smaller bars.

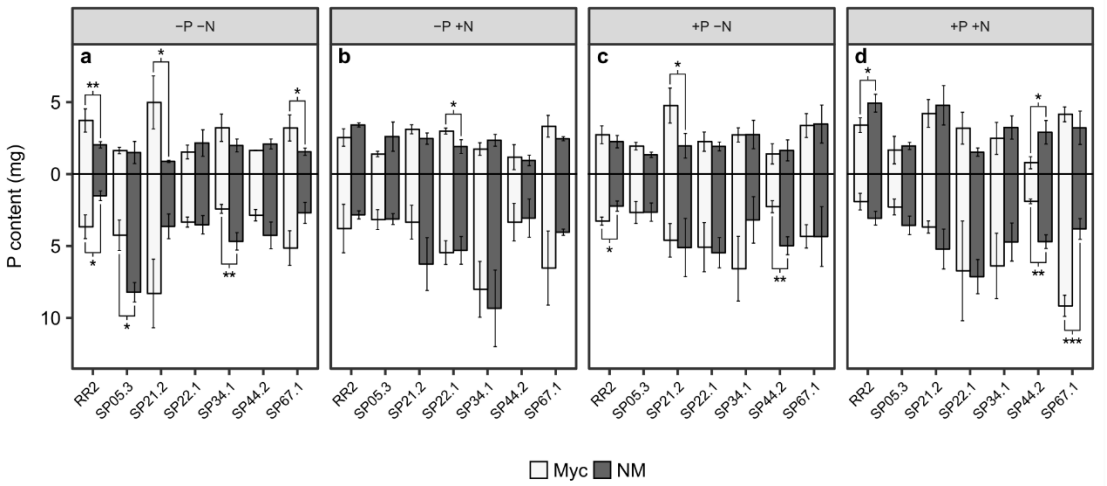

Figure 3.13. Experiment 1: mean P content value comparisons by fungal treatment. Mean P content values are presented in terms of both above ground (bars ascending from zero) and below ground (bars descending from zero) tissue values. Error bars represent the standard error of the mean (SEM). Values are partitioned according to nutrient input condition: low P and low N (A), low P and high N (B), high P and low N (C), high P and high N (D). The lighter shaded bars reflect plants inoculated with mycorrhizae (Myc) while darker shaded bars represent plants not inoculated with mycorrhizae (NM). Asterisks indicate results of independent, t-test comparisons between the mycorrhizal treatment and non-mycorrhizal control; *p*-value ≤ 0.1 (*), *p*-value ≤ 0.05 (**), *p*-value ≤ (***).

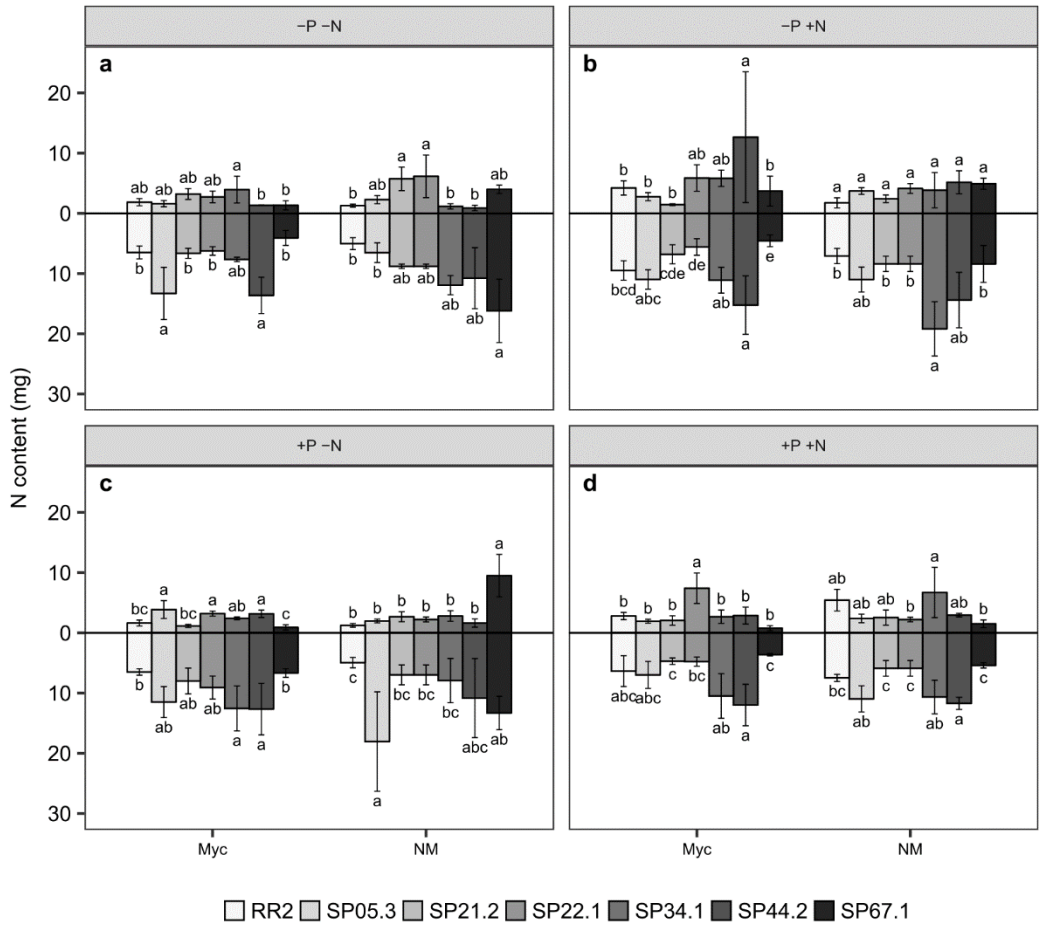

Figure 3.14. Experiment 1: mean N content value comparisons by PCG genotype. Mean N content values are presented in terms of both above ground (bars ascending from zero) and below ground (bars descending from zero) tissue values. Error bars represent the standard error of the mean (SEM). Values are partitioned according to nutrient input condition: low P and low N (A), low P and high N (B), high P and low N (C), high P and high N (D). Bar colors denote PCG genotype. Letters above and below each bar denote the results of the Fisher's least significant difference (LSD) test (p-value ≤ 0.1). LSD comparisons were made between each PCG genotype for each AM fungal treatment in nutrient partitions.

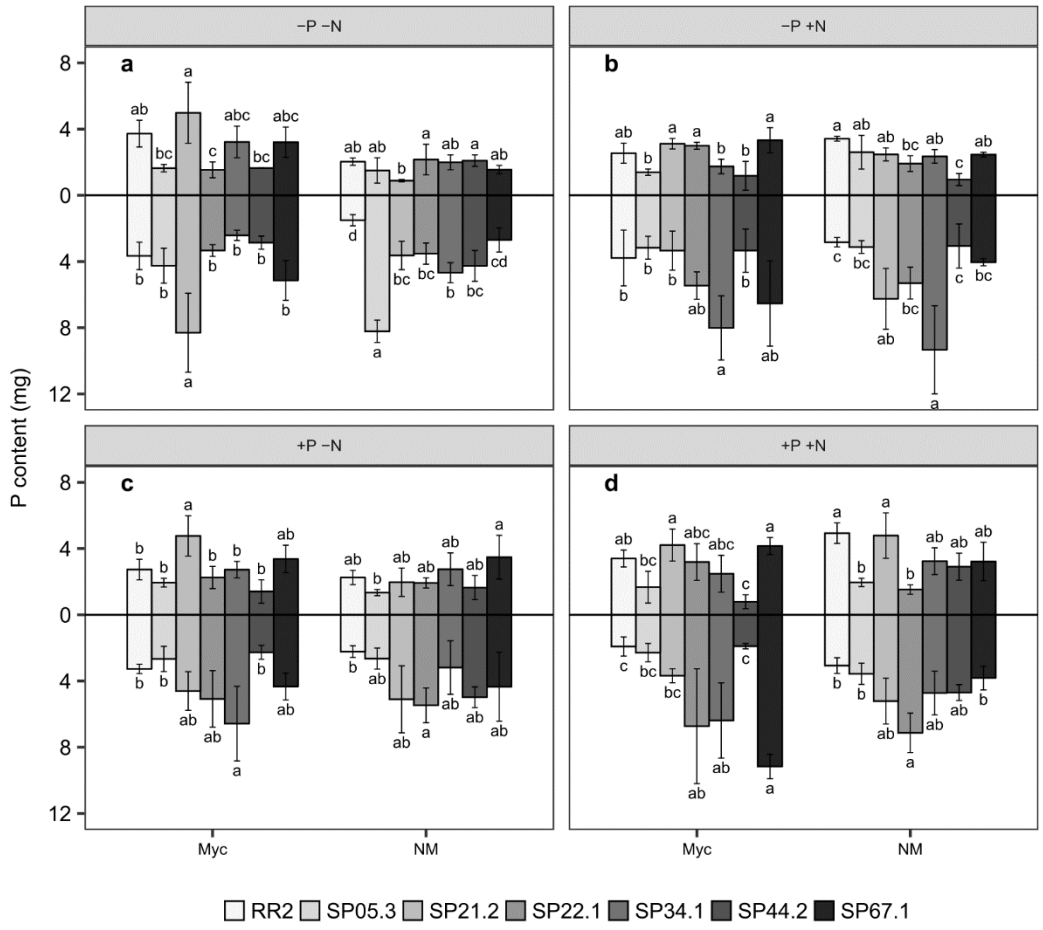

Figure 3.15. Experiment 1: mean P content value comparisons by PCG genotype. Mean P content values are presented in terms of both above ground (bars ascending from zero) and below ground (bars descending from zero) tissue values. Error bars represent the standard error of the mean (SEM). Values are partitioned according to nutrient input condition: low P and low N (A), low P and high N (B), high P and low N (C), high P and high N (D). Bar colors denote PCG genotype. Letters above and below each bar denote the results of the Fisher's least significant difference (LSD) test (p-value ≤ 0.1). LSD comparisons were made between each PCG genotype for each AM fungal treatment in nutrient partitions.

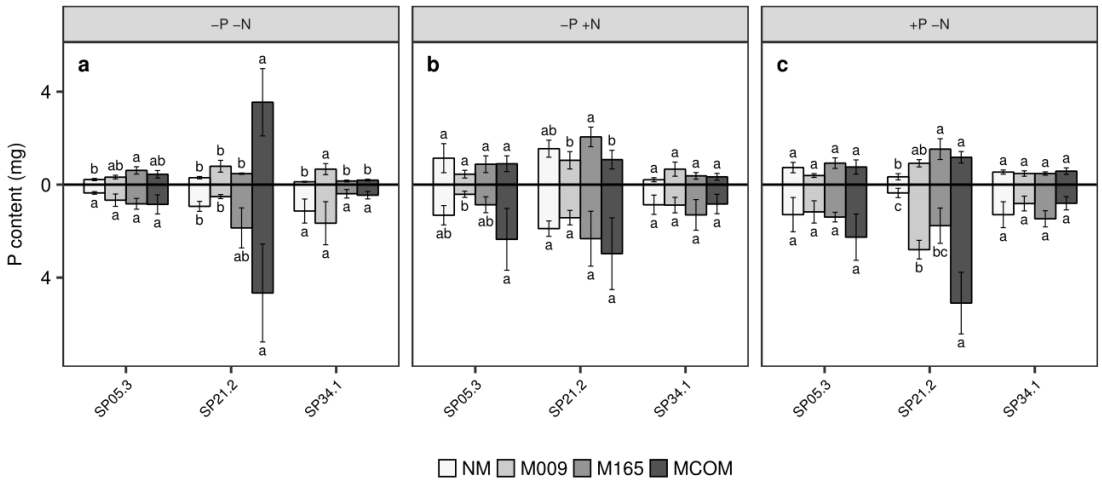

Figure 3.16. Experiment 2: mean P content value comparisons by fungal treatment. Mean P content values are presented in terms of both above ground (bars ascending from zero) and below ground (bars descending from zero) values. Error bars represent the standard error of the mean (SEM). Values are partitioned according to nutrient input conditions: low P, low N (A), low P and high N (B) and high P and low N (C). White bars indicate non-mycorrhizal plant treatments, light gray indicates *R. irregularis* isolate 009, medium gray indicates *G. aggregatum* isolate 165, and dark gray bars signify an equal combination of both 009 and 165 AM fungal isolates. Letters above and below each bar denote the results of the Fisher's least significant difference (LSD) test (p-value ≤ 0.1). LSD comparisons were made between each fungal treatment for each PCG genotype.

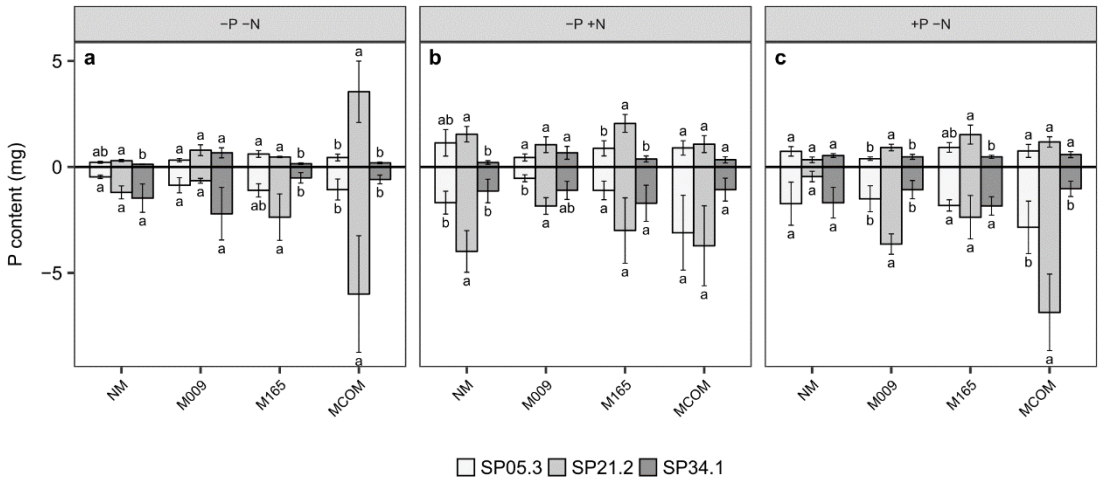

Figure 3.17. Experiment 2: mean P content value comparisons by PCG genotype. Mean P content values are presented in terms of both above ground (bars ascending from zero) and below ground (bars descending from zero) values. Error bars represent the standard error of the mean (SEM). Values are partitioned according to nutrient input conditions: low P, low N (A), low P and high N (B) and high P and low N (C). Bar color represents PCG genotype. Letters above and below each bar denote the results of the Fisher's least significant difference (LSD) test (p-value $\leq$ 0.1). LSD comparisons were made between each PCG genotype for each AM fungal treatment.

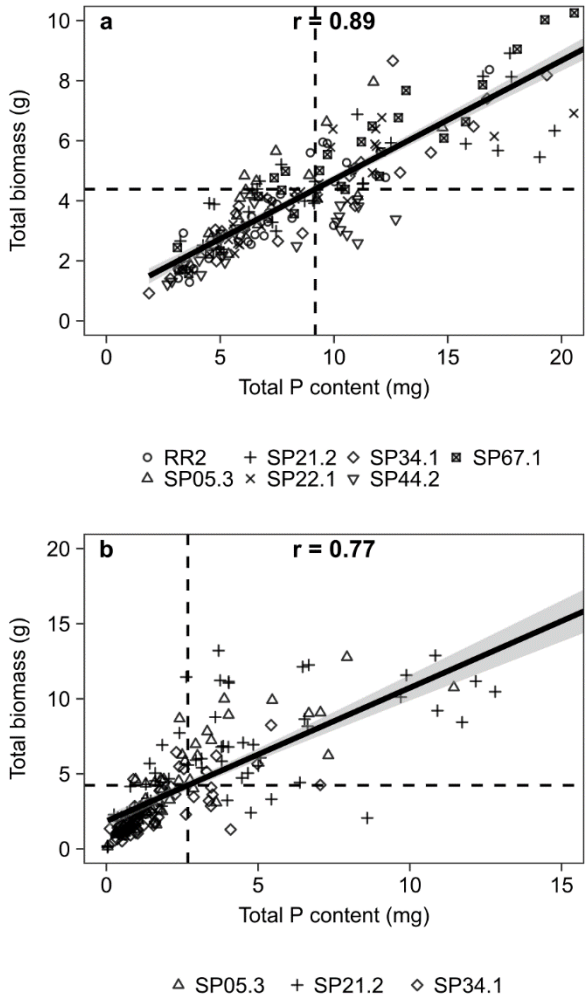

Figure 3.18. Experiment 1 & 2: linear regression of total biomass vs. P content. Comparisons between total biomass and P content were made for all mycorrhizal and non-mycorrhizal treatments in experiment 1 (A) and experiment 2 (B). Plot nodes are distinguished by their respective genotype (shape). Shaded regions signify the 95% confidence interval of the regression slope. Dotted lines represent mean values for both x and y variables.

3.6 Tables

Table 3.1. Experiment 1: ANOVA results for biomass variables. Three-way ANOVA was performed to test the effects of PCG genotype, AM fungal treatment, nutrient regimen, and their respective interactions in terms of above- and below-ground biomass. Significant values (p-value ≤ 0.05) are highlighted in bold.

	Above Ground Biomass				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	6	9.138	1.523	5.766	< **0.000**
Fungus	1	0.942	0.942	3.568	**0.061**
Nutrient	3	3.111	1.037	3.926	**0.010**
Genotype:Fungus	6	2.014	0.336	1.271	0.275
Genotype:Nutrient	18	3.875	0.215	0.815	0.680
Fungus:Nutrient	3	0.445	0.148	0.561	0.641
Genotype:Fungus:Nutrient	18	5.236	0.291	1.101	0.358
Residuals	132	34.867	0.264		
	Below Ground Biomass				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	6	69.336	11.556	4.459	< **0.000**
Fungus	1	5.516	5.516	2.129	0.147
Nutrient	3	21.803	7.268	2.804	**0.042**
Genotype:Fungus	6	22.026	3.671	1.417	0.213
Genotype:Nutrient	18	38.410	2.134	0.823	0.670
Fungus:Nutrient	3	7.113	2.371	0.915	0.436
Genotype:Fungus:Nutrient	18	23.742	1.319	0.509	0.950
Residuals	132	342.070	2.591		

Table 3.2. Experiments 1 & 2: ANOVA results for root:shoot ratios. Three-way ANOVA was performed to test the effects of PCG genotype, AM fungal treatment, nutrient regimen, and their respective interactions in terms of root:shoot ratios. Significant values (p-value ≤ 0.05) are highlighted in bold.

	Experiment 1: Root:Shoot Ratios				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	6	188.684	31.447	9.612	**< 0.000**
Fungus	1	0.034	0.034	0.010	0.919
Nutrient	3	18.890	6.297	1.925	0.129
Genotype:Fungus	6	15.295	2.549	0.779	0.588
Genotype:Nutrient	18	80.054	4.447	1.359	0.163
Fungus:Nutrient	3	2.558	0.853	0.261	0.854
Genotype:Fungus:Nutrient	18	39.694	2.205	0.674	0.832
Residuals	132	431.864	3.272		
	Experiment 2: Root:Shoot Ratios				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	2	3.455	1.727	1.555	0.215
Fungus	3	1.521	0.507	0.456	0.713
Nutrient	2	1.252	0.626	0.564	0.570
Genotype:Fungus	6	6.982	1.164	1.048	0.397
Genotype:Nutrient	4	5.160	1.290	1.161	0.331
Fungus:Nutrient	6	7.788	1.298	1.169	0.326
Genotype:Fungus:Nutrient	12	6.692	0.558	0.502	0.911
Residuals	144	159.964	1.111		

Table 3.3. Experiment 2: ANOVA results for biomass variables. Three-way ANOVA was performed to test the effects of PCG genotype, AM fungal treatment, nutrient regimen, and their respective interactions in terms of above- and below-ground biomass. Significant values (p-value ≤ 0.05) are highlighted in bold.

	Above Ground Biomass				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	2	112.081	56.041	32.964	< **0.000**
Fungus	3	46.501	15.500	9.118	< **0.000**
Nutrient	2	5.504	2.752	1.619	0.202
Genotype:Fungus	6	39.799	6.633	3.902	**0.001**
Genotype:Nutrient	4	5.043	1.261	0.742	0.565
Fungus:Nutrient	6	13.202	2.200	1.294	0.263
Genotype:Fungus:Nutrient	12	28.526	2.377	1.398	0.173
Residuals	144	244.811	1.700		
	Below Ground Biomass				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	2	87.118	43.559	14.982	< **0.000**
Fungus	3	66.262	22.087	7.597	< **0.000**
Nutrient	2	21.261	10.630	3.656	**0.028**
Genotype:Fungus	6	87.894	14.649	5.039	< **0.000**
Genotype:Nutrient	4	7.584	1.896	0.652	0.626
Fungus:Nutrient	6	8.313	1.385	0.477	0.825
Genotype:Fungus:Nutrient	12	38.455	3.205	1.102	0.363
Residuals	144	418.663	2.907		

Table 3.4. Experiments 1 & 2: ANOVA results for tiller numbers. Three-way ANOVA was performed to test the effects of PCG genotype, AM fungal treatment, nutrient regimen, and their respective interactions in terms of tiller numbers. Significant values (p-value ≤ 0.05) are highlighted in bold.

	Experiment 1: Tiller Number				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	6	45.786	7.631	8.219	< **0.000**
Fungus	1	0.085	0.085	0.091	0.763
Nutrient	3	12.849	4.283	4.613	**0.004**
Genotype:Fungus	6	7.655	1.276	1.374	0.230
Genotype:Nutrient	18	18.039	1.002	1.079	0.380
Fungus:Nutrient	3	1.921	0.640	0.690	0.560
Genotype:Fungus:Nutrient	18	18.094	1.005	1.083	0.376
Residuals	132	122.550	0.928		
	Experiment 2: Tiller Number				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	2	2.100	1.050	2.681	**0.072**
Fungus	3	0.644	0.215	0.548	0.650
Nutrient	2	2.433	1.217	3.106	**0.048**
Genotype:Fungus	6	5.189	0.865	2.208	**0.046**
Genotype:Nutrient	4	1.167	0.292	0.745	0.563
Fungus:Nutrient	6	1.789	0.298	0.761	0.602
Genotype:Fungus:Nutrient	12	4.078	0.340	0.868	0.581
Residuals	144	56.400	0.392		

Table 3.5. Experiments 1 & 2: ANOVA results for tiller weight. Three-way ANOVA was performed to test the effects of PCG genotype, AM fungal treatment, nutrient regimen, and their respective interactions in terms of above ground biomass per tiller. Significant values (p-value ≤ 0.05) are highlighted in bold.

	Experiment 1: Above Ground Biomass/Tiller				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	6	1.681	0.280	4.232	**0.001**
Fungus	1	0.050	0.050	0.762	0.384
Nutrient	3	0.276	0.092	1.390	0.249
Genotype:Fungus	6	0.562	0.094	1.415	0.214
Genotype:Nutrient	18	1.414	0.079	1.187	0.281
Fungus:Nutrient	3	0.175	0.058	0.881	0.453
Genotype:Fungus:Nutrient	18	0.945	0.052	0.793	0.706
Residuals	132	8.738	0.066		
	Experiment 2: Above Ground Biomass/Tiller				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	2	2.100	1.050	2.681	**0.072**
Fungus	3	0.644	0.215	0.548	0.650
Nutrient	2	2.433	1.217	3.106	**0.048**
Genotype:Fungus	6	5.189	0.865	2.208	**0.046**
Genotype:Nutrient	4	1.167	0.292	0.745	0.563
Fungus:Nutrient	6	1.789	0.298	0.761	0.602
Genotype:Fungus:Nutrient	12	4.078	0.340	0.868	0.581
Residuals	144	56.400	0.392		

Table 3.6. Experiments 1 & 2: ANOVA results for AM fungal root colonization. Three-way ANOVA was performed to test the effects of PCG genotype, AM fungal treatment, nutrient regimen, and their respective interactions in terms of AM fungal root colonization. Significant values (*p*-value ≤ 0.05) are highlighted in bold.

	Experiment 1: AM fungal root colonization				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	6	5261.902	876.984	3.915	**0.002**
Nutrient	3	839.677	279.892	1.249	0.299
Genotype:Nutrient	18	6684.196	371.344	1.658	**0.071**
Residuals	67	15008.438	224.007		
	Experiment 2: AM fungal root colonization				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	2	306.943	153.472	0.321	0.726
Fungus	2	611.270	305.635	0.639	0.530
Nutrient	2	1295.801	647.901	1.354	0.263
Genotype:Fungus	4	5687.897	1421.974	2.972	**0.023**
Genotype:Nutrient	4	2135.026	533.757	1.116	0.353
Fungus:Nutrient	4	1185.112	296.278	0.619	0.650
Genotype:Fungus:Nutrient	8	5975.252	746.906	1.561	0.145
Residuals	108	51674.693	478.469		

Table 3.7. Experiment 1: ANOVA results for N content variables. Three-way ANOVA was performed to test the effects of PCG genotype, AM fungal treatment, nutrient regimen, and their respective interactions in terms of above- and below-ground N content. Significant values (p-value ≤ 0.05) are highlighted in bold.

	Above Ground N Content				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	6	68.669	11.445	1.443	0.203
Fungus	1	4.252	4.252	0.536	0.465
Nutrient	3	80.943	26.981	3.403	**0.020**
Genotype:Fungus	6	136.052	22.675	2.860	**0.012**
Genotype:Nutrient	18	270.659	15.037	1.896	**0.021**
Fungus:Nutrient	3	39.251	13.084	1.650	0.181
Genotype:Fungus:Nutrient	18	224.244	12.458	1.571	**0.077**
Residuals	132	1046.709	7.930		
	Below Ground N Content				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	6	3835.632	639.272	9.136	**< 0.000**
Fungus	1	123.294	123.294	1.762	0.187
Nutrient	3	227.865	75.955	1.085	0.358
Genotype:Fungus	6	926.264	154.377	2.206	**0.046**
Genotype:Nutrient	18	1320.095	73.339	1.048	0.412
Fungus:Nutrient	3	22.299	7.433	0.106	0.956
Genotype:Fungus:Nutrient	18	765.241	42.513	0.608	0.889
Residuals	132	9236.606	69.974		

Table 3.8. Experiment 1: ANOVA results for P content variables. Three-way ANOVA was performed to test the effects of PCG genotype, AM fungal treatment, nutrient regimen, and their respective interactions in terms of above- and below-ground P content. Significant values (p-value ≤ 0.05) are highlighted in bold.

	Above Ground P Content				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	6	77.673	12.946	6.838	**< 0.000**
Fungus	1	6.735	6.735	3.558	**0.061**
Nutrient	3	22.591	7.530	3.978	**0.009**
Genotype:Fungus	6	22.357	3.726	1.968	**0.075**
Genotype:Nutrient	18	20.965	1.165	0.615	0.883
Fungus:Nutrient	3	14.689	4.896	2.587	**0.056**
Genotype:Fungus:Nutrient	18	37.897	2.105	1.112	0.348
Residuals	132	249.885	1.893		
	Below Ground P Content				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	6	375.123	62.520	4.281	**0.001**
Fungus	1	11.563	11.563	0.792	0.375
Nutrient	3	86.156	28.719	1.967	0.122
Genotype:Fungus	6	180.003	30.001	2.054	**0.063**
Genotype:Nutrient	18	278.546	15.475	1.060	0.400
Fungus:Nutrient	3	10.469	3.490	0.239	0.869
Genotype:Fungus:Nutrient	18	253.205	14.067	0.963	0.506
Residuals	132	1927.635	14.603		

Table 3.9. Experiment 2: ANOVA results for P content variables. Three-way ANOVA was performed to test the effects of PCG genotype, AM fungal treatment, nutrient regimen, and their respective interactions in terms of above- and below-ground P content. Significant values (p-value ≤ 0.05) are highlighted in bold.

	Above Ground P Content				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	2	21.926	10.963	18.535	**< 0.000**
Fungus	3	5.132	1.711	2.892	**0.037**
Nutrient	2	1.723	0.862	1.457	0.236
Genotype:Fungus	6	10.099	1.683	2.846	**0.012**
Genotype:Nutrient	4	2.867	0.717	1.212	0.308
Fungus:Nutrient	6	10.273	1.712	2.895	**0.011**
Genotype:Fungus:Nutrient	12	20.610	1.717	2.904	**0.001**
Residuals	144	85.171	0.591		
	Below Ground P Content				
	DF	**SS**	**MS**	***F*-value**	***p*-value**
Genotype	2	106.370	53.185	11.866	**< 0.000**
Fungus	3	61.115	20.372	4.545	**0.004**
Nutrient	2	14.988	7.494	1.672	0.192
Genotype:Fungus	6	88.670	14.778	3.297	**0.005**
Genotype:Nutrient	4	4.524	1.131	0.252	0.908
Fungus:Nutrient	6	19.213	3.202	0.714	0.639
Genotype:Fungus:Nutrient	12	78.845	6.570	1.466	0.144
Residuals	144	645.416	4.482		

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CHAPTER 4: TRANSCRIPTOME ANALYSIS OF PRAIRIE CORDGRASS, SWITCHGRASS, AND BRACHYPODIUM UNDER AM SYMBIOSIS

4.1 Introduction

Spartina pectinata Link, also known as prairie cordgrass (PCG), is a perennial grass that can range from sizes of 1 to more than 2 meters in height (Boe et al. 2009; Jensen 2006; Johnson et al. 2007). PCG has a broad distribution that spans throughout most the United States while also reaching all the way into the Arctic Circle of Northern Canada. PCG can grow in diverse soil conditions that include high levels of moisture (Skinner et al. 2009), well drained lands, and other stresses such as high levels of salinity (Anderson et al. 2015; Kim et al. 2011; Montemayor et al. 2008; Robben et al. 2018). Additionally, PCG has been shown to produce more biomass compared to other C4 grasses grown under similar conditions (Boe and Lee 2007; Boe et al. 2009). These traits have made PCG a prime candidate for biofuel production.

Panicum virgatum L., also known as switchgrass (SG), is another rhizomatous, perennial grass considered for bioenergy production. Like PCG, SG has a broad growing range and climatic tolerance throughout North America (Sanderson et al. 2006). SG has also been shown to have increased tolerances against pathogens, insect herbivory, and high soil moisture content (Barney et al. 2009; Hope and McElroy 1990; Saathoff et al. 2013). As a potential bioenergy resource, SG can produce significant biomass yields of more than 15 $Mg \cdot ha^{-1}$ under certain conditions (Boateng et al. 2006).

PCG and SG can form symbiotic relationships with arbuscular mycorrhizal (AM) fungi, a member of the phylum, Glomeromycota (Liepold 2013; Parrish and Fike 2005). AM symbioses occurs in ca. 65% of all terrestrial plant species including many economically important cereal crops (Smith et al. 2011) including the model grass

species, *Brachypodium distachyon* (Brachypodium). This symbiosis entails benefits for both organisms where AM fungi will obtain a fraction of the plant's carbon supplies and in return, the plant will receive numerous benefits. One particular benefit involves the increased uptake of nutrients including phosphorus, nitrogen, sulfur, magnesium, copper, and zinc (Smith et al. 2011). Other beneficial traits include increased resistance to both biotic and abiotic stresses such as pathogenicity, drought, increased saline conditions, and heavy metal contamination of soils (Jeffries et al. 2003; Newsham et al. 1995; Singh et al. 2011). Responses towards AM symbiosis can differ depending on the amount of nutrients the plant has access to, mainly, phosphorus and nitrogen. Increased phosphorus supply to plants has been reported to inhibit AM development (Abbott et al. 1984; Thomson et al. 1986). Recently, Nouri et al. (2014) have shown that both nitrogen and phosphorous are major determinants in the AM symbiosis of *Petunia hybrida*.

In the past, molecular approaches have been used to understand how the AM symbiosis is regulated in plants. Prior studies have identified genes crucial to this symbiosis including nutrient acquisition (Harrison 2012), stress resistance (Kapoor et al. 2013; Porcel et al. 2006; Weidmann et al. 2004), and AM establishment in the root cortex. DGE analysis in roots of mycorrhizal plants compared to non-mycorrhizal plants has been performed in key systems including *Medicago truncatula* (Liu et al. 2007), *Lotus japonicus* (Guether et al. 2009) , and tomato (*Solanum lycopersicum*) (Güimil et al. 2005). While roots may be the main area of observation for AM symbiosis, systemic effects can also be observed in leaves and shoots of plants. So far, differential gene expression (DGE) analyses of this phenomenon have been performed in tomato

(Cervantes-Gámez et al. 2016), *M. truncatula* (Adolfsson et al. 2017; Bonneau et al. 2013), and pedunculate oak trees (Kurth et al. 2015).

With the advent high-throughput methodologies like RNA-seq, massive amounts of DGE data can be obtained in a short amount of time from more samples and uncharacterized plants when compared to microarray-based studies. DGE studies have been performed in PCG to investigate responses to salt stress (Robben et al. 2018) and cold stress (Nah et al. 2016). Studies in SG have investigated heat stress (Li et al. 2013), development (Palmer et al. 2015), and drought response (Hivrale et al. 2016). As of this date, no DGE studies have investigated responses to mycorrhizal symbiosis in either of these plants in relation to above- and below-ground plant material. In this study we seek to investigate the systemic responses of PCG and SG to the AM symbiosis under varying nitrogen and phosphorus levels in comparison to Brachypodium. The objectives of this study are to (1) identify genes and pathways that are differentially regulated in response to AM symbiosis in leaf material and (2) identify potential response variances in expression under high and low level nutrient conditions with phosphate and nitrogen.

4.2 Materials and Methods

4.2.1 Plant material and fungal culture

We generated plant replicates by growing seedlings from either PCG (*Spartina pectinata* Link "Red River" germplasm collection), SG (*Panicum virgatum* "Kanlow" cultivar) or Brachypodium (*Brachypodium distachyon* strain Bd21) seed material. Seeds from the prior grasses were first sterilized in 12.73 mM sodium dichloroisocyanurate (NaDCC) solution for 1.5 h. Next, we placed seeds on moist, sterilized germination paper in a covered petri dish and placed them in a Conviron TC30 growth chamber (ca. 25° C, 14 h day^{-1}). Seeds were germinated for one week and transferred to individual Magenta GA-7 growth vessels containing 25 grams of sterile perlite moistened with 20 mL of sterile Type 1 H_2O.

We used axenic mycorrhizal root organ cultures (ROC) of Ri T-DNA-transformed *Daucus carota* roots (clone DCI) to produce the fungal inoculum, *Rhizophagus irregularis* DAOM 197198 (RI) (Schüßler and Walker 2010). The mycorrhizal root systems were grown on mineral medium at 27° C (Bécard and Fortin 1988) for approximately 8 weeks. Sterile spores were isolated by blending the medium in 10 μM sodium citrate buffer at pH 6 for 1 minute. The solution was passed through a 22 μm filter paper, residual root material was manually removed, and the remaining spores were transferred into 250 mL of ultrapurified water.

4.2.2 Experimental design

Seedlings were initially fertilized with a modified Ingestad's nutrient solution (Ingestad 1960). Phosphate and nitrogen concentrations were altered to simulate marginal soil conditions found in Eastern South Dakota based off of soil analysis from this area

(Liepold 2013). The total nutrient profile was as follows: 1 mM NH_4NO_3, 0.05 mM KH_2PO_4, 0.617 mM KCl, 1 mM $CaCl_2{\cdot}2H_2O$, 0.015 mM Fe-Ethylenediaminetetraacetic acid (EDTA), 0.625 mM $MgSO_4{\cdot}7H_2O$, 0.003 mM $MnCl_2{\cdot}4H_2O$, 0.016 mM H_3BO_3, 0.113 μM Zn-EDTA, 0.372 μM $CuCl_2{\cdot}2H_2O$, and 0.034 μM $Na_2MoO_4{\cdot}2H_2O$. After initial fertilization, we inoculated half of the replicates from each grass with ca. 300 spores in 1.5 mL of RI spore solution. The other half were inoculated with an equal volume of sterile filtration solution to account for any nutrient variability. After 3 weeks, plants were subjected to modified nutrient treatments. One half of the plants were inoculated with another treatment of the prior fertilization solution. The other half of the plants were fertilized with the modified Ingestad's solution using further reduced phosphorus and nitrogen concentrations (0.15 mM and 7.5 μM, respectively). Plants were monitored for water loss every two days by weighing a random sample of plant systems from each species. Each nested treatment had 6 biological replicates which were organized in a randomized block design. Plants were grown for an additional 5 weeks and were harvested.

4.2.3 AM colonization analysis and RNA extraction

At the end of the treatment period, leaf and root material were excised and flash frozen in liquid nitrogen. Samples were stored at -80° C until the next step. Before flash freezing, an aliquot of the fresh root material from each mycorrhizal treatment was excised and analyzed for AM colonization. Root aliquots were initially fixed in 50% ethanol (v/v) for 72 h at room temperature. The fixed root material was washed with 10% potassium hydroxide solution (w/v) at 90° C for 1.5 h, rinsed three times with tap water and stained with 0.1% Chlorazol Black E (w/v) in lacto-glycerol (lactic

acid:glycerol:water; 13:12:16, v:v:v) for ca. 16 h at room temperature. After the staining, roots were transferred into lacto-glycerol and stored until further analysis. To quantify the AM fungal colonization rate, a modified grid-line intersect method was used (Giovannetti and Mosse 1980; McGonigle et al. 1990)

Total RNA from leaf material of three biological replicates from each treatment was extracted using TRIzol reagent (Ambion, Carlsbad, CA) following the manufacturer's instructions. RNA quality was determined by running an aliquot of each sample on a 1% agarose gel. RNA concentrations were estimated using a ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Samples were sent out for cDNA library prep and Illumina HiSeq sequencing (Joint Genome Institute, Walnut Creek, California).

4.2.4 Alignments and de novo assembly

Sequences from each sample were imported into CLC v9.5.2 (Qiagen, Hilden, Germany) for sequence quality analysis, alignments, and assemblies. Raw reads were trimmed with a Phred quality score ≥ 27 and a maximum number of ambiguous nucleotides of 2. High quality Brachypodium and SG reads were mapped to the v3.1 and v1.1 reference genomes, respectively. Since PCG does not have a reference genome, reads were constructed into a de novo transcriptome assembly. High quality PCG reads were then mapped back to the contig assembly to create a count array. Finally, BLAST was used to align the PCG contigs to *Sorghum bicolor* v3.1 DOE-JGI cds data (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Sbicolor).

4.2.5 RNA-seq analysis and functional annotation

To determine differential gene expression, raw count reads were imported into R and analyzed using edgeR v2.3 (Robinson et al. 2010). After initial QC analysis (Figure 4.1), differential expression was determined using data normalized via trimmed means of M-values (Robinson and Oshlack 2010) and pairwise performed using the Exact Test. Multiple testing correction was implemented using false discovery rate for adjusted *p*-values. Transcript IDs or contigs with a total value of ≤ 100 reads were filtered from the initial count matrix. Functional annotation and pathway analysis of differentially expressed IDs was analyzed using MapMan v3.6.0 (Thimm et al. 2004)

4.3 Results

4.3.1 Mycorrhizal colonization

In this study, we investigated the changes in gene expression caused by AM fungal symbiosis in three grass species subjected to two nutrient regimens. Grass seedlings were inoculated with RI (Myc) and non-mycorrhizal (NM) controls were inoculated with sterile *D. carota* root material. At the time of harvest, average mycorrhizal colonization for all plants was 46.86% ± 8.21. No colonization could be observed in the NM controls. Total RNA from leaf material was then extracted and sequenced.

4.3.2 Read metrics and de novo assembly overview

After trimming and filtering, the total number of reads remained consistent for each treatment. The number of reads for Brachypodium and SG ranged from ca. 48 - 60 million average read counts (Table 4.1 and Table 4.2). PCG reads were slightly higher with average reads ranging from ca. 57 - 63 million (Table 4.3). All PCG reads were then assembled into contigs which totaled 220,366 with a median length of 911 bp (Table 4.4).

4.3.3 RNA-seq overview

DEGs were determined by comparing the expression levels of each grass species under Myc and NM conditions for high (HPHN) and low (LPLN) input nutrient regimens. These comparisons were conducted in edgeR (Robinson et al. 2010) in which significantly expressed genes were filtered out and reported (Table 4.5). Brachypodium had the highest number of DEGs for both nutrient regimens while SG and PCG had the lower. Each grass species showed higher numbers of up-regulated mycorrhizal responsive DEGs under LPLN treatments. Brachypodium and SG displayed higher numbers of down-regulated DEGs under HPHN treatments.

4.3.4 Significant DEG activity

Next, significant DEGs for each treatment were analyzed in terms of highest absolute fold change values. Significant DEGs identified in Brachypodium under LPLN conditions were involved in transferase activity (up-regulated) and protein kinase activity (down-regulated). Under HPHN conditions, DEGs were up-regulated for exocyst development, carrier-protein synthase, and transferase activity (Table 4.6). Significant DEGs in SG under LPLN conditions were involved in transferase activity (up-regulated) and transcription factor development (down-regulated). Under HPHN conditions, two up-regulated genes were involved in proteinase complex development and disease resistance proteins. One down-regulated gene was involved in carbohydrate binding (Table 4.7). Under LPLN conditions, we identified one contig as an up-regulated gene involved in exocyst development. Two down regulated contigs were involved in transcription factor activity and chloroplast development. Under HPHN conditions, one up-regulated contig was found to be closely related to a gene involved in glycine degradation. Two down-regulated contigs were likely genes involved in lipoxygenase and signaling activity (Table 4.8).

4.3.5 Carbohydrate metabolism

Since no PCG contigs defined to *S. bicolor* successfully annotated to carbohydrate metabolism, only Brachypodium and SG were analyzed. In Brachypodium treatments, various carbohydrate metabolic pathways showed mostly up-regulated and down-regulated DEG activity in LPLN and HPHN conditions, respectively (Figure 4.2 and Table 4.9). SG showed up-regulated activity in both nutrient conditions. SG in LPLN conditions displayed more transcripts with down-regulated activity compared to the

Brachypodium LPLN treatment (Figure 4.3 and Table 4.10). Despite this, both showed up-regulation in sucrose synthase activity.

4.3.6 Photosynthetic pathways

Like carbohydrate metabolism, no PCG contigs defined to *S. bicolor* successfully annotated to identifiers related to photosynthesis. Under LPLN and HPHN conditions, Brachypodium showed mostly down regulation of a few transcripts (Figure 4.4 and Table 4.11). Conversely, SG typically displayed up-regulated DEGs under both nutrient treatments. Compared to the Brachypodium samples, SG had more DEGs map to photosynthetic pathways under LPLN treatments (Figure 4.5 and Table 4.12).

4.3.7 Abiotic and biotic stress

Stress pathway-related DEGs had the highest counts in both Brachypodium and SG. Brachypodium under LPLN conditions showed more up-regulation compared to DEGs identified in the HPHN treatment (Figure 4.6 and Table 4.13). SG, however, did not show this same trend. More up-regulated DEGs were identified in the HPHN condition while a more equal number of up- and down-regulated DEGs were identified in the LPLN treatment (Figure 4.7 and Table 4.14). 4 DEGs in PCG were also mapped to these pathways. Two DEGs under LPLN conditions were up- and down-regulated while the other two were both down-regulated under the HPHN treatment (Figure 4.8 and Table 4.15). While numerous stress-related responses were identified in each treatment combination, combinations disease- and pathogen-related defense genes were found to be either up- or down-regulated under the two nutrient conditions for each grass species.

4.3.8 Hormone metabolism

All three grass species had varying numbers of DEGs that mapped to hormone metabolic pathways. Under LPLN conditions, Brachypodium displayed a majority of up-regulated DEGs compared to HPHN conditions (Table 4.16). Amongst these up-regulated DEGs, several were identified to be involved in jasmonic acid synthesis (Figure 4.9). While SG had fewer DEGs map to hormone pathways, more DEGs were up-regulated under low nutrient conditions than its high nutrient counterpart (Table 4.17). Although one DEG was identified to be involved in jasmonic acid production, it was slightly down-regulated (Figure 4.10). Similar to Brachypodium and SG, PCG also showed similar regulatory trends, albeit, in limited numbers (Table 4.18).

4.3.9 Transporters

DEGs related to various transporter activities were also identified in each grass species. Following prior trends, Brachypodium showed higher numbers of DEGs compared to the other grasses. In LPLN conditions, many of these mapped DEGs were up-regulated while more DEGs were identified to be down-regulated in the HPHN treatment (Table 4.19). More DEGs in both SG treatments were shown to be up-regulated compared to Brachypodium (Table 4.20). Similar to prior classifications, PCG had the lowest number of mapped DEGs (Table 4.21). In general, a wide variety of transporters were expressed under mycorrhizal conditions. These include mainly nutrient-based and sugar-based transporters.

4.4 Discussion

We have shown differential gene expression under mycorrhizal conditions for three grass species under two different nutrient conditions. The number of mycorrhizal-responsive DEGs varied from 26 to 473. This number is generally lower than what has been reported in leaf tissue of other plant species. Cervantes-Gámez et al. (2016) reported 724 mycorrhizal-responsive genes in the leaves of mycorrhizal tomato plants. Liu et al. (2007) reported 599 genes in the leaves of *Medicago truncatula*. Fiorilli et al. (2009) identified 422 genes in the shoots of tomato shoots. This number is closest to what we identified in Brachypodium.

Some of the most significantly up-regulated DEGs reported in Brachypodium, SG, and PCG under low input conditions were found to be *GT2*, *LGT4*, and *EXO*, respectively. *GT2* and *LGT4* are glycosyltransferases and are involved in xyloglucan and glycan biosynthesis (Plaza et al. 2014). *EXO* is a gene required for the production of the protein, exordium, which aids in cell expansions in leaves (Schröder et al. 2009). Coincidentally, genes similar to the reported glycosyltransferases have been found to be up-regulated in leaves of other plants during AM symbiosis (Adolfsson et al. 2017). Kurth et al. (2015) were also able to show up-regulation of glycosyltransferases and increased *EXO* regulation in Pedunculate oak trees. Conversely, the most significantly down-regulated DEGs reported in the three grass species under low nutrient conditions included genes necessary for protein kinases (*MHK*), transcription factors (*BZIP53*), and interestingly, superoxide dismutase (*SFD3*). Cervantes-Gámez et al. (2016) were also able to report down-regulation in bZIP transcription factors in leaves of mycorrhizal tomato plants. In this paper they proposed that negative regulation of these specific

transcription factors is a response to colonization of root systems. *MHK* gene is a potential homolog to mitogen-activated protein kinases. These enzymes can be involved in a wide variety of signal transduction pathways. These include response to abiotic and biotic stress, cell development, and hormone regulation (Ichimura et al. 2002). Kinases have also been shown to be regulated in plant leaves during mycorrhizal symbiosis (Kurth et al. 2015). Superoxide dismutase, a product of *SFD3,* is an antioxidant-related enzyme which can be invoked during times of stress (Ruiz-Lozano et al. 2012). Regulation of *SFD3* and other superoxide dismutase-related genes has been shown to up- and down-regulated in prior studies. For example, Talaat and Shawky (2011) reported up-regulation of plants grown under saline-stress, while Liu and Wu (2014) have shown the opposite. This variation can also be attributed to different fungal species and host plants (Ruiz-Lozano et al. 2012).

When additional phosphate and nitrogen is added to the plant systems, a different range of DEGs is shown. Significantly up-regulated DEGs reported in each grass species are responsible for defensive measures including, exocyst development (*SEC3A*), glycine cleavage (*GLDP2*), and proteasome development (PBF1). While exocysts play primary roles in plant development and cytokinesis (Zhang et al. 2013), recent work has shown it may play a role in plant defense. Du et al. (2018) showed that proper exocyst development is needed for defense against plant pathogens in *Nicotiana benthamiana*. It has also been reported that *SEC3A* in rice is crucial for plant immunity and defense responses (Ma et al. 2017). Enhanced glycine decarboxylase (*GLDP2*) activity has been shown in *Arabidopsis thaliana* to help prevent the pathogenic effects of harpin protein via induction of the nitric oxide pathway (Palmieri et al. 2010). Up-regulation of various

proteasome subunits has been shown to be involved in enhanced defense and survival rates of tomato plants against *Pseudomonas syringae* (Üstün et al. 2016). Significantly down-regulated genes are involved in lipoxygenase activity (*LOX1*), chaperone molecules for superoxide dismutase activation (*CPN21*), and f-box protein development (*PP2-B12*). Similar to what was described for regulation under low nutrient conditions, superoxide dismutases have been found to be either up- or down-regulated in different AM symbioses. *CPN21* and *PP2-B12* activity has shown to be involved in mycorrhizal responsive DEGs in the leaves of tomato plants (Cervantes-Gámez et al. 2016).

Carbohydrate metabolism was primarily up-regulated in Brachypodium and partially in SG during AM symbiosis under low input conditions. This is expected since AM fungi are obligate biotrophs that require carbon resources from the host plant. Of the up-regulated DEGs observed, sucrose synthase was identified in both plants under low nutrient conditions. It is commonly believed that one of the main sources of carbon transferred to AM fungi is in the form of sucrose (Smith and Smith 2011). Increased sucrose synthesis has also been observed in mycorrhized Norway spruce and aspen trees (Loewe et al. 2000). While SG did show some up-regulation of carbohydrate related transcripts under high nutrient conditions, Brachypodium showed mainly down-regulated DEGs. These DEGs were primarily involved in sucrose production.

Since AM fungi require carbon resources from the host plant, increased photosynthetic activity within responsive plants should also occur due to increased levels of P and N acquisition (Kaschuk et al. 2009; Tsimilli-Michael et al. 2000). Our results for Brachypodium indicate limited numbers of photosynthetic-related DEGs for both nutrient input conditions. The number of these DEGs for SG were much higher for low input

conditions but around the same amounts for the high nutrient treatment when compared to Brachypodium. The results for SG coincide with findings by Zouari et al. (2014). In this study, many genes were found to be up-regulated in terms of photosynthetic activity in tomato plants and fruits. Interestingly, the Brachypodium results mimic those found in Cervantes-Gámez et al. (2016).

AM fungi are also known to provide plants resistance to abiotic and biotic stress (Al-Karaki and Al-Raddad 1997; Sharifi et al. 2007). Our results show a wide variety of up- and down-regulated mycorrhizal responsive DEGs in each treatment condition. In each plant, many disease resistance-related genes were identified. In Brachypodium and SG, several WRKY related genes were found to be up-regulated. WRKY transcription factors are activated in the last part of amplification cascades caused by various MPK genes. WRKY genes have been shown to play a role for plant immunity (Pandey and Somssich 2009). Under low nutrient conditions, Brachypodium showed several up-regulated DEGs related to peroxidase activity. Peroxidases have been shown to detoxify reactive oxygen species which can cause cell damage (Caverzan et al. 2012). Additionally, ethylene-related DEGs were also found to regulated in both SG and Brachypodium. Ethylene has also been identified in plant defense responses (Ecker and Davis 1987). Pozo and Azcón-Aguilar (2007) have also suggested that ethylene regulation is involved in AM symbioses. Interestingly, under higher nutrient conditions, more DEGs appear to be down-regulated. This is most notable in Brachypodium and SG treatments.

Hormonal metabolism in plant systems is crucial for stress regulation and mycorrhizal symbiosis. In the root systems, this is needed for mycorrhization to occur

(Foo et al. 2013). Our results also indicate a systemic hormonal response in grass leaves that can fluctuate based on nutrient inputs. We have identified multiple oxidoreductases, aldo/keto reductases, transferases, ethylene response factors, and precursors related to jasmonic acid (JA) signaling. The JA signaling pathway has been shown to play defensive roles in protecting plants against herbivory and necrotrophic pathogens. This pathway has also been shown to be activated during mycorrhizal symbiosis events (Carvalhais et al. 2013). JA signaling was found to be up-regulated under low nutrient conditions in Brachypodium but was not identified in SG or PCG. The Brachypodium results echo what was identified in prior work conducted by Cervantes-Gámez et al. (2016).

A multitude of transporters were also identified in each grass species. Indeed, this is expected since symbiotic plant interactions with AM fungi require the shuttling of resources to and from the root interfacial apoplast (Harrison 1999; Smith et al. 2011). Our RNA-seq data has shown up-regulated transporter activity involved in potassium, ammonia, and nitrate in Brachypodium under low nutrient conditions. Putative potassium and ammonia transporters have previously been recognized in mycorrhizal root systems (Breuillin-Sessoms et al. 2015; Garcia and Zimmermann 2014). In contrast, there is currently no available information related to similar transporters in the leaves of plants. Cervantes-Gámez et al. (2016) were also able to show the presence of putative up-regulated ammonia transporters in tomato leaves. These data suggest that nitrogen and potassium taken up by transporters in the roots can be shuttled to the leaves of grass responding to mycorrhizal infection using similar transporters. Sugar transporters including sucrose (*SUT2*) were identified throughout each grass species under both

nutrient conditions. Many of these transporters were up-regulated which suggests the importance of sugar transport in leaves as well as root systems. Sucrose is a common form of sugar for long-distance transport in plants. Expression profiling of *Medicago truncatula* have indicated that sucrose transporters including *SUT2* in leaves are involved in AM symbiosis (Doidy et al. 2012; Kafle et al. 2018).

The exchange of carbon resources and mineral nutrients between plant and AM fungi is key to regulation of the AM symbiosis. When plants receive sufficient nutrients, carbon transfer to AM fungi can be limited and vice-versa. Olsson et al. (2002) have shown that once sufficient levels of P is supplied to a plant, C releases to the AM fungi is reduced. Nagy et al. (2009) have shown that down-regulation of mineral transporters in the root can also occur. We have shown variations in transcriptomes of three mycorrhizal grass species under low- and high-input nutrient conditions. Changes to carbohydrate metabolism, photosynthesis, sugar transporters, nutrient transporters, and response to disease signaling were most notably observed between these two nutrient conditions. Similar changes have been observed in root profiles of *Populus trichocarpa* when subjected to different N and P input regimens (Calabrese et al. 2017) and *Medicago truncatula* (Bonneau et al. 2013). Our data implies that response to AM symbiosis under different nutrient conditions can also have a systemic effect observable in areas of the plant other than root material. This data may potentially establish essential framework of how potential grass feedstocks can interact with AM fungi under variable nutrient conditions.

4.5 Future Work

Certain caveats, however, need to be discussed. Since this project is dealing with three separate plant systems, relative comparisons in transcript fold changes besides classifying a transcript/contig as up- or down-regulated becomes problematic. Normalization was implemented only to take into account for variability of library size and not directly observing contig or transcript ID length. Therefore, reads per kilobase of transcript, per million mapped reads (RPKM) should also be investigated. This is most notable for length variabiliity within the contigs of PCG (Table 4.4). While we were able to discuss certain aspects of regulation in PCG, this is compounded by the fact that we observed minmal differential expression in each of the two nutrient input systems. This could be attributed to the fact that we had to use a de novo assembly in which (1) an assembly must be constructed, (2) contigs must be mapped back to the assembly, and (3) differentially expressed contigs must annotated to a closely related species. For (3), not all contigs will have significant alignment to the reference species.

Additionally, while this data may provide insight into how these plants can respond to AM fungi, further studies for this project should be conducted to corroborate these findings. Since we have only observed systemic responses in leaf material, additional DGE research should be conducted in other tissues, including the root system.

4.6 Figures

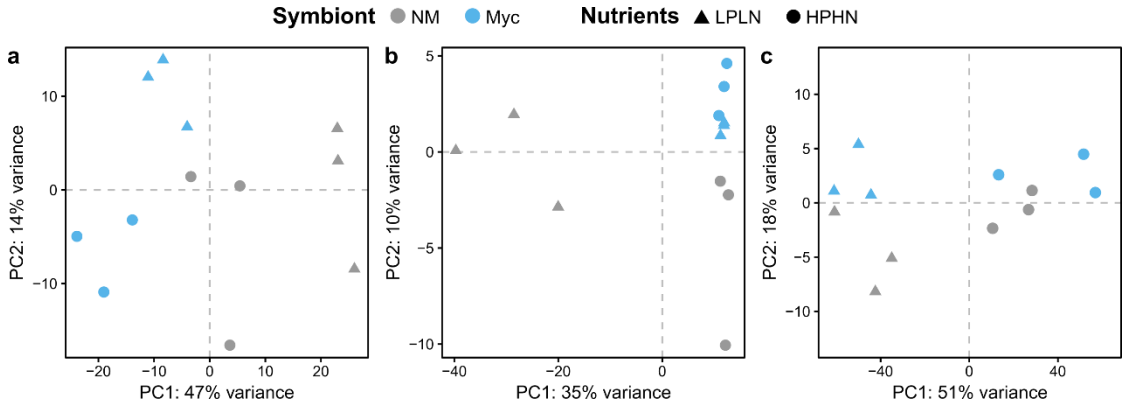

Figure 4.1 Principal component analysis of each grass species. Principal component analysis was performed on Brachypodium (a), switchgrass (b), and prairie cordgrass (c) to determine variance between each sample and their respective treatment. LPLN refers to low phosphorus and nitrogen nutrition. HPHN refers to high phosphorus and nitrogen nutrition. "Myc" signifies samples treated with the AM fungus, *R. irregularis* DAOM197198. "NM" signifies the non-mycorrhizal control treatment.

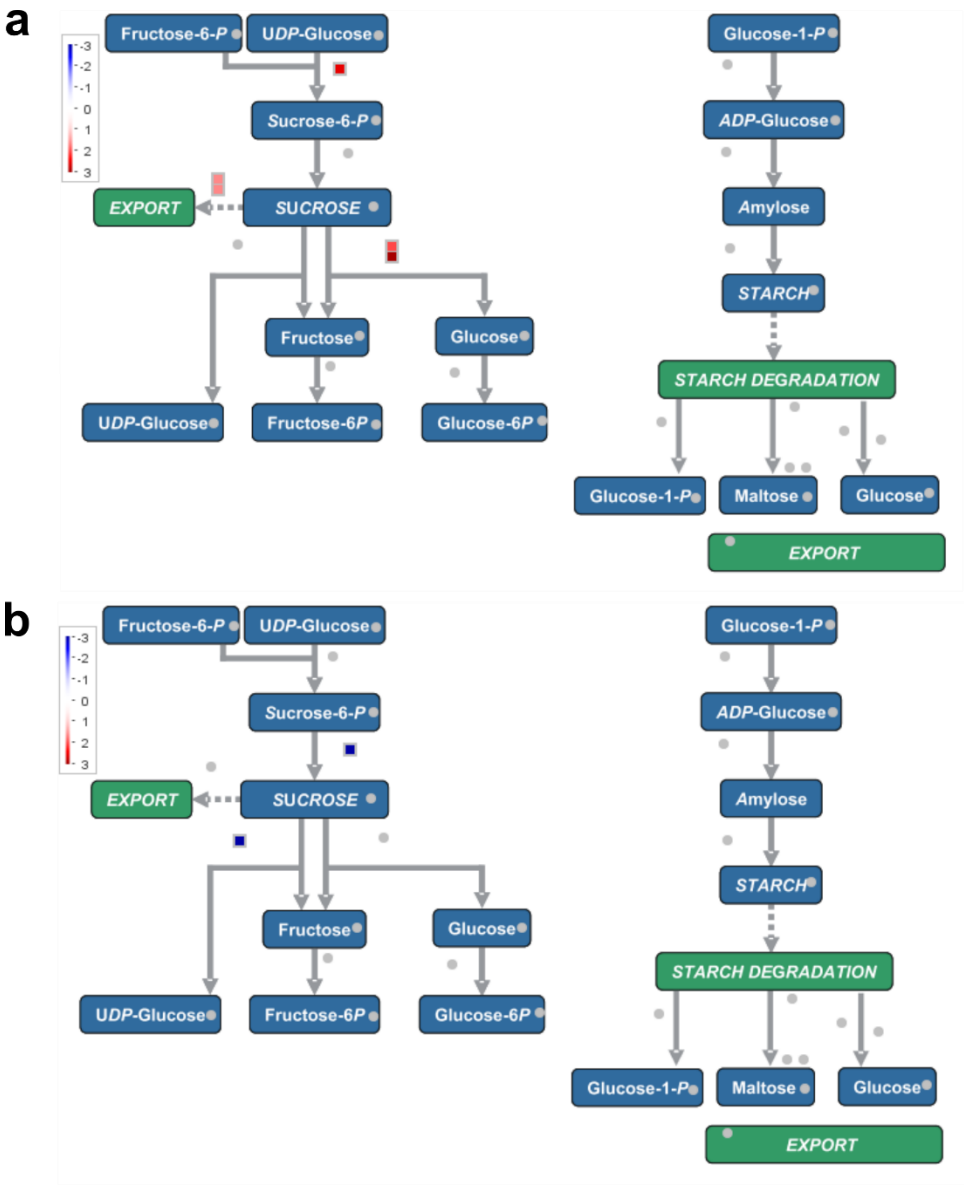

Figure 4.2. Sucrose and starch metabolic pathways of Brachypodium differential gene expression under (a) low and (b) high nutrient inputs. Mycorrhiza-responsive genes according to their RNA-seq fold changes are shown above. Pathways indicate samples treated with either (a) low phosphorus and nitrogen and (b) high phosphorus and nitrogen nutrient inputs. Colored boxes indicate transcripts annotated to their respective function. Red and blue colors indicate up- and down-regulation, respectively. Grey dots indicate that no IDs were mapped to a particular pathway location.

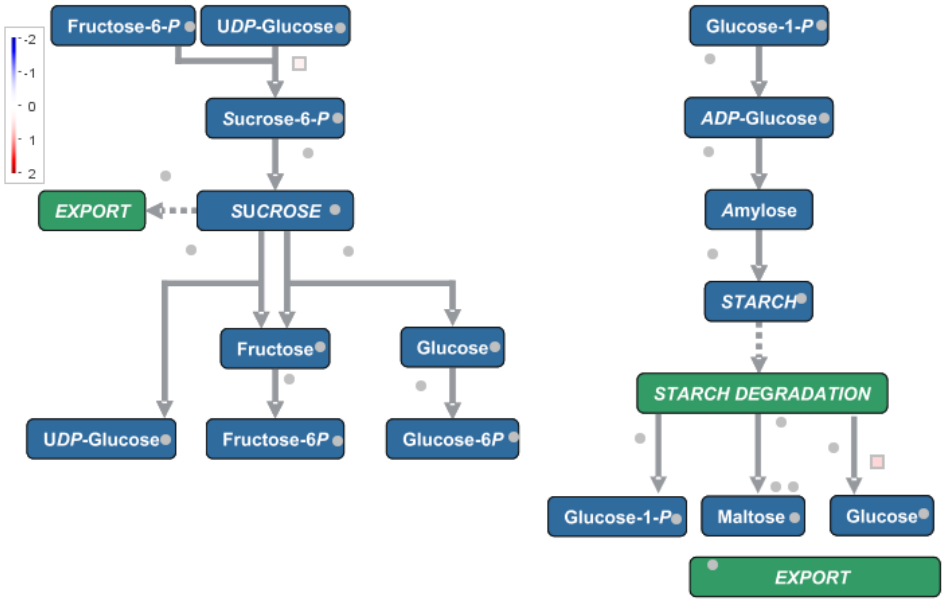

Figure 4.3. Sucrose and starch metabolic pathways of switchgrass differential gene expression under only high nutrient inputs. Mycorrhiza-responsive genes according to their RNA-seq fold changes are shown above. Pathways indicate samples treated with high phosphorus and nitrogen nutrient inputs. Colored boxes indicate transcripts annotated to their respective function. Red and blue colors indicate up- and down-regulation, respectively. Grey dots indicate that no IDs were mapped to a particular pathway location.

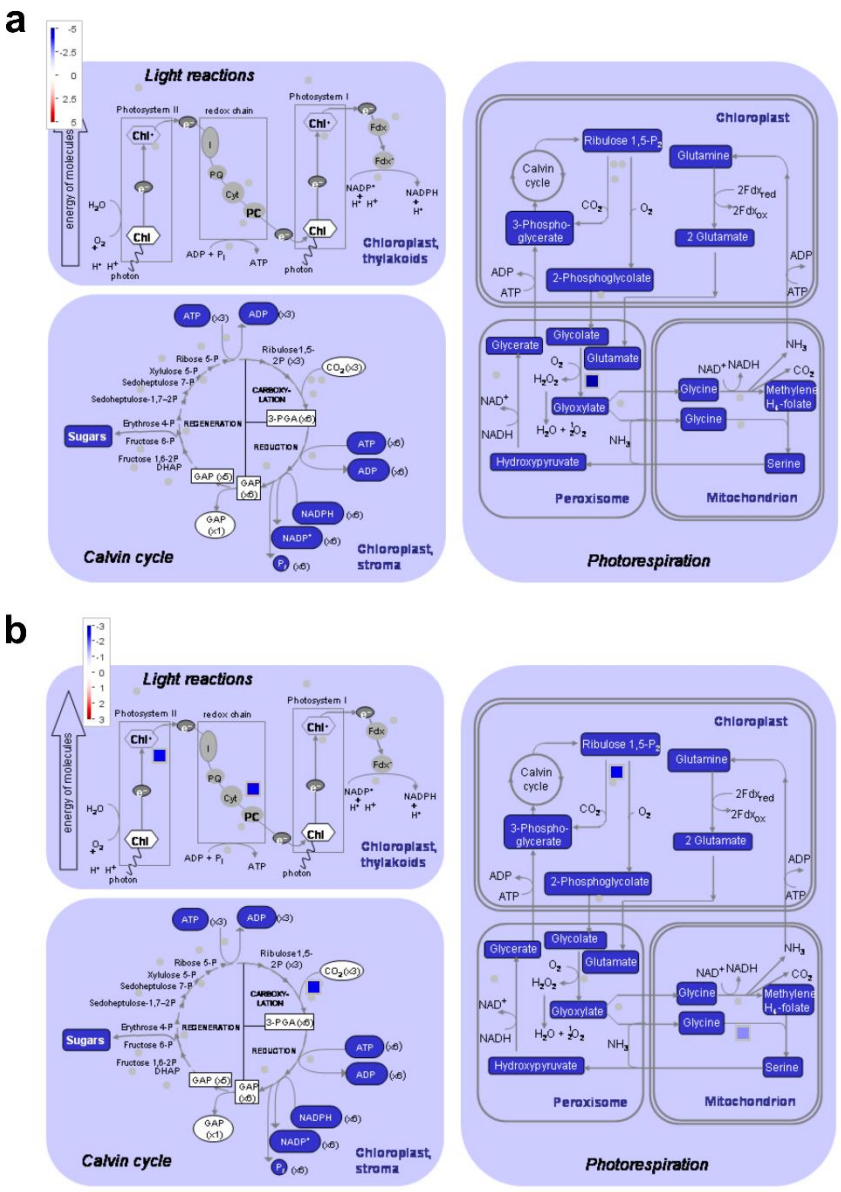

Figure 4.4. Photosynthesis pathways of Brachypodium differential gene expression under (a) low and (b) high nutrient inputs. Mycorrhiza-responsive genes according to their RNA-seq fold changes are shown above. Pathways indicate samples treated with either (a) low phosphorus and nitrogen and (b) high phosphorus and nitrogen nutrient inputs. Colored boxes indicate transcripts annotated to their respective function. Red and blue colors indicate up- and down-regulation, respectively. Grey dots indicate that no IDs were mapped to a particular pathway location.

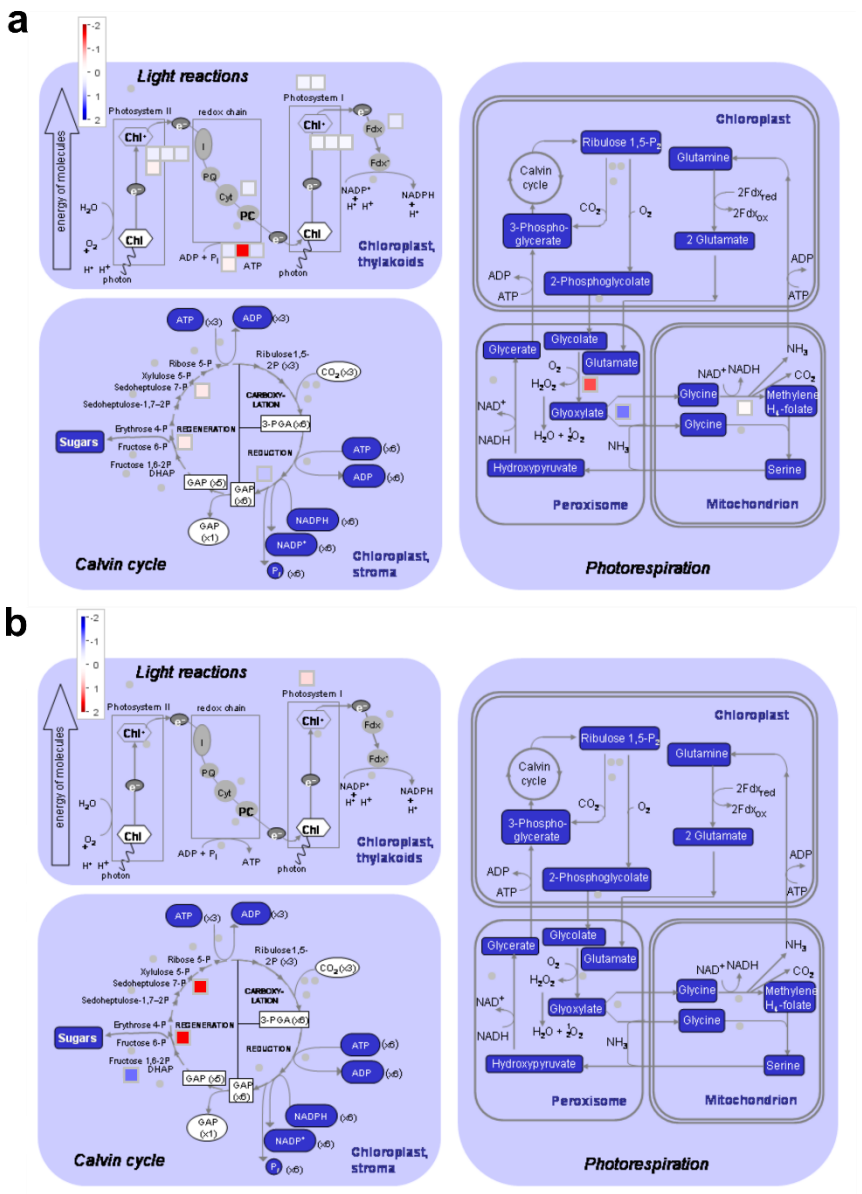

Figure 4.5. Photosynthesis pathways of switchgrass differential gene expression under (a) low and (b) high nutrient inputs. Mycorrhiza-responsive genes according to their RNA-seq fold changes are shown above. Pathways indicate samples treated with either (a) low phosphorus and nitrogen and (b) high phosphorus and nitrogen nutrient inputs. Colored boxes indicate transcripts annotated to their respective function. Red and blue colors indicate up- and down-regulation, respectively. Grey dots indicate that no IDs were mapped to a particular pathway location.

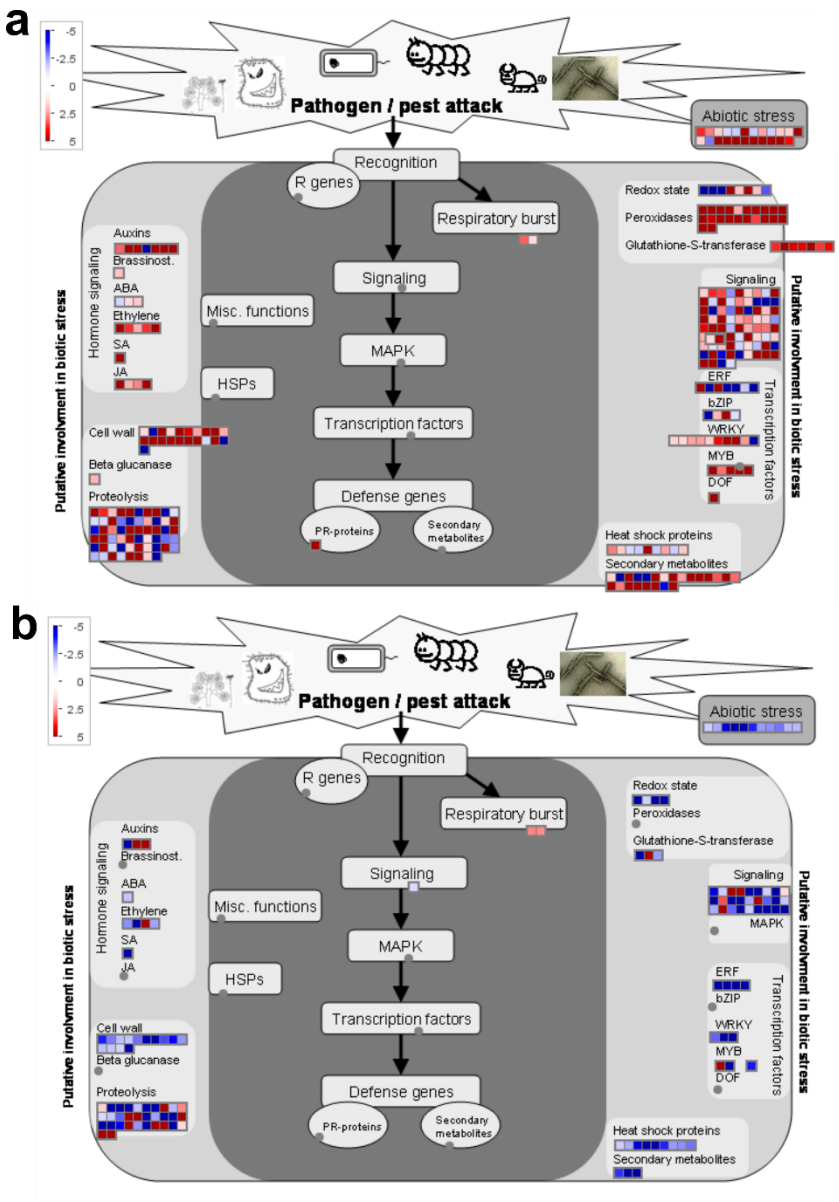

Figure 4.6. Abiotic and biotic stress pathways of Brachypodium differential gene expression under (a) low and (b) high nutrient inputs. Mycorrhiza-responsive genes according to their RNA-seq fold changes are shown above. Pathways indicate samples treated with either (a) low phosphorus and nitrogen and (b) high phosphorus and nitrogen nutrient inputs. Colored boxes indicate transcripts annotated to their respective function. Red and blue colors indicate up- and down-regulation, respectively. Grey dots indicate that no IDs were mapped to a particular pathway location.

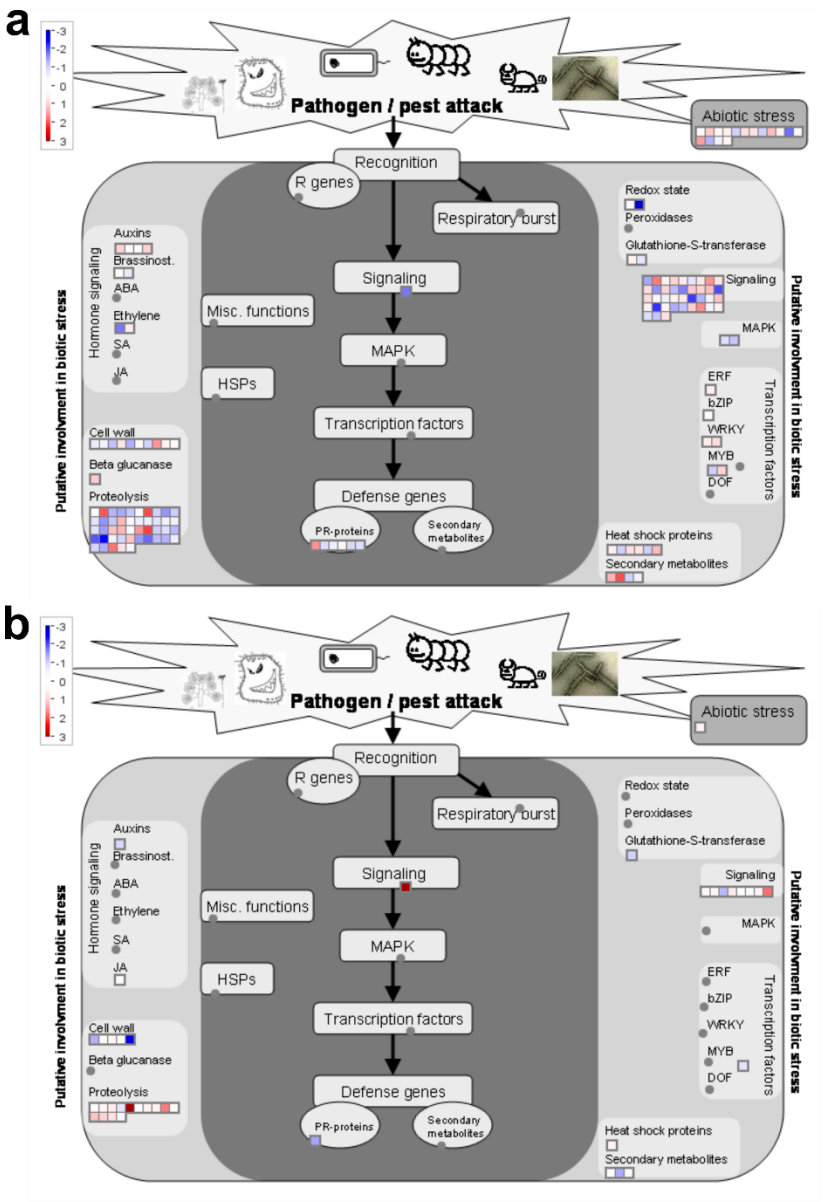

Figure 4.7. Abiotic and biotic stress pathways of switchgrass differential gene expression under (a) low and (b) high nutrient inputs. Mycorrhiza-responsive genes according to their RNA-seq fold changes are shown above. Pathways indicate samples treated with either (a) low phosphorus and nitrogen and (b) high phosphorus and nitrogen nutrient inputs. Colored boxes indicate transcripts annotated to their respective function. Red and blue colors indicate up- and down-regulation, respectively. Grey dots indicate that no IDs were mapped to a particular pathway location.

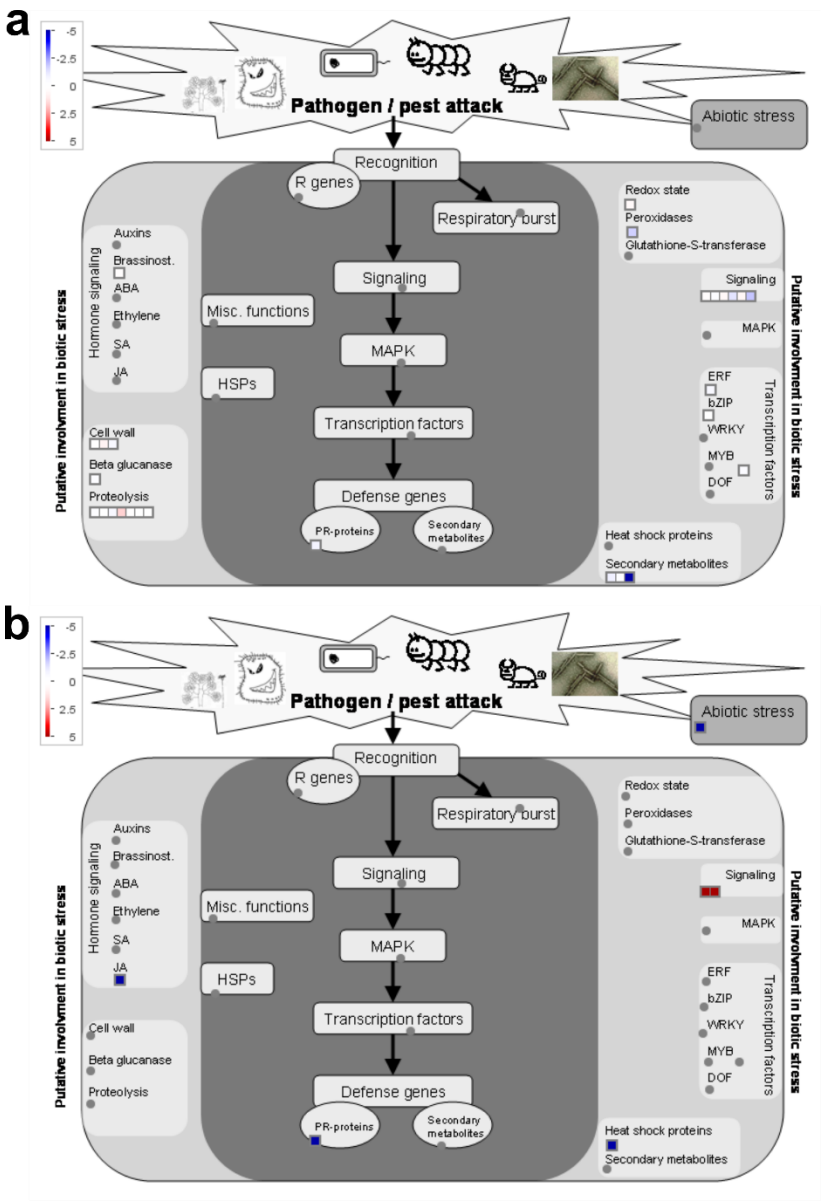

Figure 4.8. Abiotic and biotic stress pathways of prairie cordgrass differential gene expression under (a) low and (b) high nutrient inputs. Mycorrhiza-responsive genes according to their RNA-seq fold changes are shown above. Pathways indicate samples treated with either (a) low phosphorus and nitrogen and (b) high phosphorus and nitrogen nutrient inputs. Colored boxes indicate contigs annotated to their respective function. Red and blue colors indicate up- and down-regulation, respectively. Grey dots indicate that no IDs were mapped to a particular pathway location.

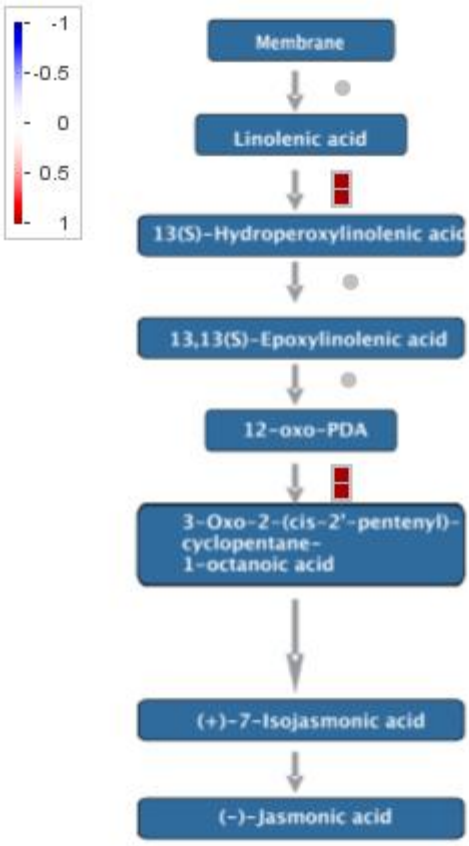

Figure 4.9. Jasmonic acid synthesis pathways of Brachypodium differential gene expression under only low nutrient inputs. Differentially expressed mycorrhiza-responsive genes according to their RNA-seq fold changes are shown above. Pathways indicate samples treated with low phosphorus and nitrogen nutrient inputs. Colored boxes indicate transcripts annotated to their respective function. Red and blue colors indicate up- and down-regulation, respectively. Grey dots indicate that no IDs were mapped to a particular pathway location.

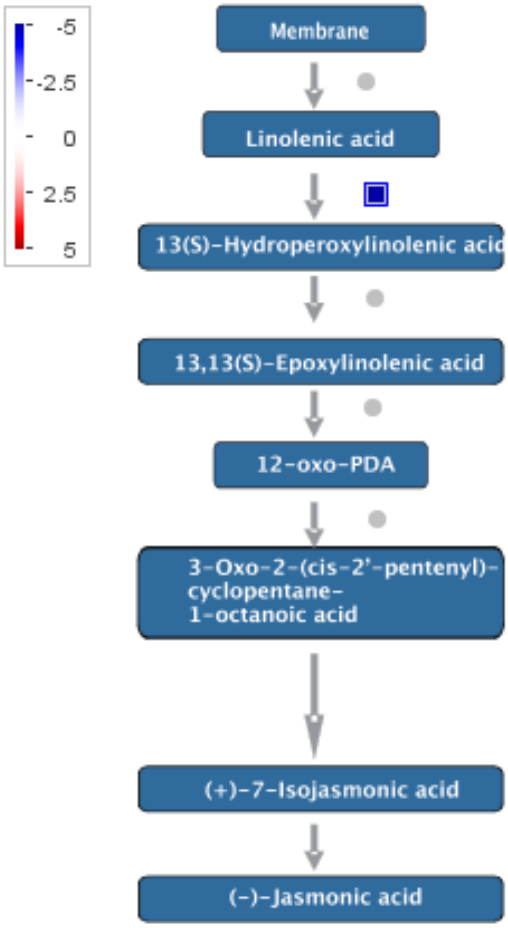

Figure 4.10. Jasmonic acid synthesis pathways of Brachypodium differential gene expression under only low nutrient inputs. Mycorrhiza-responsive genes according to their RNA-seq fold changes are shown above. Pathways indicate samples treated with low phosphorus and nitrogen nutrient inputs. Colored boxes indicate contigs annotated to their respective function. Red and blue colors indicate up- and down-regulation, respectively. Grey dots indicate that no IDs were mapped to a particular pathway location.

4.7 Tables

Table 4.1. Metrics of Brachypodium RNA-seq reads. Brachypodium (Brach) sequence reads are shown for replicates in each treatment. Additionally, mean ($\bar{x}$), and standard deviation (**SD**) are calculated for each treatment.

Plant	**Nutrient**	**Symbiont**	**Rep.**	**Reads**	$\bar{x}$	**SD**
Brach	LPLN	Myc	1	41,430,496	51,276,182	10,429,812
			2	50,192,542		
			3	62,205,508		
		NM	1	55,596,566	49,939,750	4,899,713
			2	47,198,038		
			3	47,024,646		
	HPHN	Myc	1	67,267,275	54,447,711	11,270,440
			2	49,978,777		
			3	46,097,081		
		NM	1	44,982,282	49,837,202	4,379,897
			2	51,037,549		
			3	53,491,776		

Note 1: LPLN: low phosphorus and nitrogen nutrition

HPHN: high phosphorus and nitrogen nutrition

Note 2: Myc: Mycorrhizal treatment with *R. irregularis* DAOM 197198

NM: Non-mycorrhizal control

Table 4.2. Metrics of switchgrass RNA-seq reads. Switchgrass (SG) sequence reads are shown for replicates in each treatment. Additionally, mean ($\bar{x}$), and standard deviation (**SD**) are calculated for each treatment.

Plant	**Nutrient**	**Symbiont**	**Rep.**	**Reads**	$\bar{x}$	**SD**
SG	LPLN	Myc	1	52,180,530	53,230,359	4,817,243
			2	49,024,606		
			3	58,485,942		
		NM	1	60,605,390	50,049,527	9,180,485
			2	45,615,170		
			3	43,928,020		
	HPHN	Myc	1	62,721,452	59,866,615	8,240,641
			2	50,578,179		
			3	66,300,214		
		NM	1	41,163,025	48,331,169	9,867,964
			2	44,244,518		
			3	59,585,965		

Note 1: LPLN: low phosphorus and nitrogen nutrition

HPHN: high phosphorus and nitrogen nutrition

Note 2: Myc: Mycorrhizal treatment with *R. irregularis* DAOM 197198

NM: Non-mycorrhizal control

Table 4.3. Metrics of prairie cordgrass RNA-seq reads. Prairie cordgrass (PCG) sequence reads are shown for replicates in each treatment. Additionally, mean ($\bar{x}$), and standard deviation (**SD**) are calculated for each treatment.

Plant	**Nutrient**	**Symbiont**	**Rep.**	**Reads**	$\bar{x}$	**SD**
PCG	LPLN	Myc	1	58,510,228	57,689,379	6,247,497
			2	51,072,034		
			3	63,485,876		
		NM	1	64,256,150	57,017,977	7,619,756
			2	57,731,016		
			3	49,066,764		
	HPHN	Myc	1	66,281,492	63,352,339	15,309,691
			2	46,789,694		
			3	76,985,832		
		NM	1	60,208,710	61,463,531	7,221,685
			2	54,951,488		
			3	69,230,396		

Note 1: LPLN: low phosphorus and nitrogen nutrition

HPHN: high phosphorus and nitrogen nutrition

Note 2: Myc: Mycorrhizal treatment with *R. irregularis* DAOM 197198

NM: Non-mycorrhizal control

Table 4.4. Contig metrics of PCG de novo assembly. Contig measurements are reported as upper quartile (N75), median (N50), lower quartile (N25), minimum, maximum and average lengths in bp. Total number of contigs used for this project is also shown (Count).

Measurement	**Value**
N75	528
N50	911
N25	1,676
Minimum	181
Maximum	26,212
Average	771
Count	220,366

Table 4.5. Differentially expressed genes overview. Total numbers of differentially expressed genes are shown for each mycorrhizal versus non-mycorrhizal treatment. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition. Up: up-regulated genes (fold change > 0), Down: down-regulated genes (fold change < 0).

Plant	**Nutrient**	**Up**	**Down**	**Total**
Brachypodium	LPLN	279	194	473
	HPHN	136	145	281
PCG	LPLN	82	56	138
	HPHN	12	14	26
SG	LPLN	136	41	177
	HPHN	83	59	142

Table 4.6. Top 10 most differentially expressed genes in Brachypodium. Genes denoted by Brachypodium transcript name did not contain a gene symbol in the annotation file. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition.

LPLN	**HPHN**
Bradi2g04430.1	SEC3A
GT2	KAS III
Bradi1g51555.1	NHL8
Bradi5g14097.1	Bradi2g50830.9
Bradi3g43390.1	Bradi5g10954.2
Bradi3g48815.4	Bradi4g30075.3
Bradi3g16300.1	Bradi3g57700.1
MHK	Bradi1g52551.1
Bradi4g45166.4	Bradi2g56537.6
Bradi1g48257.7	Bradi4g27456.6

Table 4.7. Top 10 most differentially expressed genes in switchgrass. Genes denoted by SG transcript name did not contain a gene symbol in the annotation file. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition.

LPLN	**HPHN**
Pavir.5KG553700.1	Pavir.3KG101900.13
LGT4	PBF1
Pavir.7NG235000.1	RPP13
Pavir.J057500.1	Pavir.7NG178400.5
Pavir.9NG280700.1	Pavir.9KG159600.5
Pavir.J457100.1	Pavir.2KG078800.3
Pavir.9NG127200.3	PP2-B12
Pavir.8KG374200.1	Pavir.3KG466100.1
BZIP53	Pavir.2KG541400.2
Pavir.3NG054100.1	Pavir.7KG013100.2

Table 4.8. Top 10 most differentially expressed genes in prairie cordgrass. Genes denoted by PCG contig ID did not contain a gene symbol or failed to be annotated by the *Sorghum bicolor* annotations. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition.

LPLN	**HPHN**
contig_101525	contig_146965
contig_051804	contig_133249
contig_046310	contig_117108
contig_076964	contig_065325
EXO	GLDP2
contig_099242	LOX1
contig_100137	CPN21
contig_063469	contig_016656
AHL1	contig_064609
FSD3	contig_071643

Table 4.9. Functional classification of Brachypodium transcript IDs related to carbohydrate metabolism. Significant differentially expressed mycorrhiza-responsive trancript IDs according to their RNA-seq fold changes are shown below. Functional descriptions are based on IDs annotated to MapMan bins 2 and 3. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition.

Nutrient	ID	Description	Fold Change
LPLN	Bradi1g60950.1	trehalose-6-phosphate phosphatase	3.35
	Bradi4g21750.1	sucrose phosphate synthase	2.58
	Bradi2g12427.1	beta-fructofuranosidase	2.05
	Bradi3g46600.1	beta-fructofuranosidase 5	1.22
	Bradi5g12280.1	aldose 1-epimerase family protein	-1.71
HPHN	Bradi2g36350.2	sucrose-phosphatase 1	-1.09
	Bradi4g29030.1	trehalose-6-phosphate phosphatase	-4.03
	Bradi1g29570.1	sucrose synthase 6	-7.27

Table 4.10. Functional classification of switchgrass transcript IDs related to carbohydrate metabolism. Significant differentially expressed mycorrhiza-responsive trancript IDs according to their RNA-seq fold changes are shown below. Functional descriptions are based on IDs annotated to MapMan bins 2 and 3. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition.

Nutrient	ID	Description	Fold Change
LPLN	Pavir.7KG035300.1	alpha-amylase	1.67
	Pavir.4KG096200.1	sucrose synthase	0.66
	Pavir.1KG310000.1	starch branching enzyme 2.2	0.53
	Pavir.8KG156300.1	carbohydrate kinase	-0.79
	Pavir.3NG191300.1	hydrolase	-0.89
	Pavir.3NG181500.1	haloacid dehalogenase	-1.14
	Pavir.5KG170400.1	3'(2'),5'-bisphosphate nucleotidase	-1.17
	Pavir.9KG108800.1	kinase	-1.29
HPHN	Pavir.2KG542300.1	4-alpha-glucanotransferase	0.64
	Pavir.1KG090700.1	sucrose phosphate synthase 1F	0.38
	Pavir.3NG191300.1	hydrolase	0.25

Table 4.11. Functional classification of Brachypodium transcript IDs related to photosynthesis. Significant differentially expressed mycorrhiza-responsive trancript IDs according to their RNA-seq fold changes are shown below. Functional descriptions are based on IDs annotated to MapMan bin 1. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition.

Nutrient	**ID**	**Description**	**Fold Change**
LPLN	Bradi1g20870.1	(S)-2-hydroxy-acid oxidase	-1.65
HPHN	Bradi2g56557.1	serine hydroxymethyltransferase 7	0.41
	Bradi3g27266.1	NAD(P)H dehydrogenase subunit H	-1.42
	Bradi2g13100.1	NADH dehydrogenase D3	-3.23
	Bradi2g20990.1	large subunit of RUBISCO	-3.4
	Bradi3g17187.1	cytochrome f apoprotein	-5.4
	Bradi4g07440.1	one helix protein	-8.09

Table 4.12. Functional classification of switchgrass transcript IDs related to photosynthesis. Significant differentially expressed mycorrhiza-responsive trancript IDs according to their RNA-seq fold changes are shown below. Functional descriptions are based on IDs annotated to MapMan bin 1. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition.

Nutrient	ID	Description	Fold Change
LPLN	Pavir.3KG272800.1	alanine:glyoxylate aminotransferase	4.75
	Pavir.5KG416500.1	phosphoribulokinase	3.47
	Pavir.1KG073700.1	glyceraldehyde-3-phosphate dehydrogenase	3.11
	Pavir.4NG323400.1	ATP synthase delta chain-related	2.36
	Pavir.6KG235800.1	FED A	1.94
	Pavir.3KG266600.1	ATP synthase	1.9
	Pavir.5KG212200.1	CP12-2	1.84
	Pavir.1NG318300.1	oxygen evolving enhancer 3	1.66
	Pavir.1KG154500.1	photosynthetic electron transfer c	1.61
	Pavir.2KG502000.1	photosystem II subunit X	1.59
	Pavir.2NG339500.1	chlorophyll binding protein	1.3
	Pavir.2KG409700.1	photosystem I subunit G	1.23
	Pavir.5KG476000.1	glycine decarboxylase P-protein 2	-0.86
	Pavir.3NG010500.1	ATPase F subunit	-1.44
	Pavir.5NG635000.1	(S)-2-hydroxy-acid oxidase	-5.4
	Pavir.1NG331300.1	formyltetrahydrofolate deformylase	-7.45
HPHN	Pavir.4KG017300.1	transketolase	6.55
	Pavir.2KG506700.1	chlorophyll binding protein	2.42
	Pavir.5KG128900.1	fructose-bisphosphate aldolase	-4.89

Table 4.13. Functional classification of Brachypodium transcript IDs related to abiotic and biotic stress. Significant differentially expressed mycorrhiza-responsive trancript IDs according to their RNA-seq fold changes are shown below. Functional descriptions are based on IDs annotated to MapMan bin 20. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition.

Nutrient	ID	Description	Fold Change
LPLN	Bradi4g03270.2	disease resistance protein	18.73
	Bradi3g15220.1	germin-like protein	14.61
	Bradi4g03270.1	disease resistance protein	7.37
	Bradi3g21597.1	protein ankyrin protein kinase	5.71
	Bradi2g39460.1	disease resistance protein	5.44
	Bradi4g00610.1	HOPZ-activated resistance	-2.29
	Bradi5g23182.1	wound-response protein	-2.92
	Bradi2g18840.1	disease resistance protein	-3.03
	Bradi4g39317.1	pathogen resistance protein	-1.9
	Bradi1g57400.1	thionin DB4 precursor	-7.54
HPHN	Bradi2g43880.1	expressed protein	4.39
	Bradi2g52450.1	disease resistance protein	3.85
	Bradi2g19090.3	respiratory burst oxidase protein F	2.77
	Bradi2g19090.1	respiratory burst oxidase protein F	2.73
	Bradi1g34380.1	disease resistance protein	2.33
	Bradi3g58590.1	heat shock protein	-4.5
	Bradi5g02037.1	heat shock protein	-8.71
	Bradi1g53850.1	heat shock protein	-8.91
	Bradi2g60260.1	leucine rich protein	-6.46
	Bradi4g12770.1	disease resistance protein	-9.29

Table 4.14. Functional classification of switchgrass transcript IDs related to abiotic and biotic stress. Significant differentially expressed mycorrhiza-responsive trancript IDs according to their RNA-seq fold changes are shown below. Functional descriptions are based on IDs annotated to MapMan bin 20. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition.

Nutrient	ID	Description	Fold Change
LPLN	Pavir.8KG088200.1	lipase	1.72
	Pavir.9KG265900.1	red chlorophyll catabolite reductase	1.69
	Pavir.2NG134600.1	disease resistance protein	1.59
	Pavir.1KG445500.1	dehydration-responsive protein	1.5
	Pavir.1KG087900.1	CASEIN LYTIC PROTEINASE B4	1.24
	Pavir.9KG406700.1	transferase	-1.05
	Pavir.5KG698400.1	dehydration-responsive protein	-1.32
	Pavir.1KG316500.1	zinc ion binding protein	-1.77
	Pavir.5KG611800.1	dehydration-responsive protein	-1.83
	Pavir.1KG097900.1	pathogen resistance protein	-2.05
HPHN	Pavir.1KG335400.1	calmodulin binding protein	3.22
	Pavir.1KG073200.1	pathogen resistance protein	2.58
	Pavir.9KG406700.1	transferase	0.54
	Pavir.2KG043200.1	heat shock protein	0.52
	Pavir.1KG097900.1	pathogen resistance protein	0.51
	Pavir.1KG137600.1	disease resistance protein	-1.43

Table 4.15. Functional classification of prairie cordgrass transcript IDs related to abiotic and biotic stress. Significant differentially expressed mycorrhiza-responsive contigs according to their RNA-seq fold changes are shown below. Functional descriptions are based on IDs annotated to MapMan bin 20. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition.

Nutrient	**ID**	**Description**	**Fold Change**
LPLN	Sobic.002G189100.1	glycosyl hyrdolase	0.89
	Sobic.005G220200.1	disease resistance protein	-0.85
HPHN	Sobic.005G220200.1	disease resistance protein	-5.83
	Sobic.003G101500.1	DNAJ heat shock N-terminal protein	-6.59

Table 4.16. Functional classification of Brachypodium transcript IDs related to hormone metabolism. Significant differentially expressed mycorrhiza-responsive trancript IDs according to their RNA-seq fold changes are shown below. Functional descriptions are based on IDs annotated to MapMan bin 17. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition.

Nutrient	ID	Description	Fold Change
LPLN	Bradi3g48190.1	oxidoreductase	5.69
	Bradi3g26900.1	aldo/keto reductase	4.19
	Bradi4g34760.1	protein calmodulin binding protein	3.72
	Bradi1g47850.1	2OG-Fe(II) oxygenase	3.57
	Bradi3g44210.1	Auxin-responsive SAUR gene family member	2.82
	Bradi1g42760.1	S-adenosylmethionine-dependent methyltransferase	2.76
	Bradi2g11610.1	universal stress protein	2.5
	Bradi2g28150.1	Glycine and cysteine rich family protein	2.41
	Bradi3g35060.1	auxin-responsive protein,	2.26
	Bradi1g05870.1	12-oxophytodienoate reductase	1.41
HPHN	Bradi4g41377.1	growth regulator protein	4.26
	Bradi4g37080.1	O-fucosyltransferase	3.7
	Bradi5g04340.1	oxidoreductase	1.98
	Bradi5g16310.1	ABA-responsive protein	-0.57
	Bradi5g19100.1	1-aminocyclopropane-1-carboxylate synthase	-0.77
	Bradi2g06670.1	gibberellin 2-beta-dioxygenase	-0.86
	Bradi4g16110.1	S-adenosyl-L-methionine:carboxyl methyltransferase	-1.41
	Bradi2g19900.1	gibberellin 2-beta-dioxygenase	-2.08
	Bradi1g09877.1	IAA-Leu conjugate hydrolase	-2.37

Table 4.17. Functional classification of switchgrass transcript IDs related to hormone metabolism. Significant differentially expressed mycorrhiza-responsive trancript IDs according to their RNA-seq fold changes are shown below. Functional descriptions are based on IDs annotated to MapMan bin 17. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition.

Nutrient	**ID**	**Description**	**Fold Change**
LPLN	Pavir.5KG074800.1	O-fucosyltransferase	1.04
	Pavir.3KG223700.1	phosphoinositide binding protein	1.01
	Pavir.5KG048800.1	DEAD box RNA helicase	0.69
	Pavir.7KG358600.1	aluminium induced protein	0.31
	Pavir.1KG553100.1	calmodulin binding protein	0.3
	Pavir.5KG022800.1	jasmonate-amino synthetase	-0.33
	Pavir.1KG043800.1	cycloartenol synthase	-0.71
	Pavir.1KG547200.1	ethylene binding protein	-1.75
HPHN	Pavir.1KG334000.1	12-oxophytodienoate reductase 2	-0.27
	Pavir.3KG058600.1	O-fucosyltransferase	-0.94

Table 4.18. Functional classification of prairie cordgrass transcript IDs related to hormone metabolism. Significant differentially expressed mycorrhiza-responsive contigs according to their RNA-seq fold changes are shown below. Functional descriptions are based on IDs annotated to MapMan bin 17. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition.

Nutrient	**ID**	**Description**	**Fold Change**
LPLN	Sobic.008G142400.5	cycloartenol synthase 1	0.34
HPHN	Sobic.003G385500.1	lipoxygenase 1	-6.46

Table 4.19. Functional classification of Brachypodium transcript IDs related to transport. Significant differentially expressed mycorrhiza-responsive trancript IDs according to their RNA-seq fold changes are shown below. Functional descriptions are based on IDs annotated to MapMan bin 34. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition.

Nutrient	ID	Description	Fold Change
LPLN	Bradi1g72910.1	peptide transporter 2	7.95
	Bradi1g76640.2	potassium ion transmembrane transporter	7.56
	Bradi4g26342.1	tetracycline:hydrogen antiporter	7.52
	Bradi3g08690.1	carbohydrate transmembrane transporter	7.29
	Bradi1g08060.1	calcium channel protein	7.06
	Bradi5g17690.1	ammonia transporter	6.98
	Bradi3g08557.2	mitochondrial substrate carrier protein	6.97
	Bradi3g01250.1	nitrate transmembrane transporter	6.68
	Bradi5g12030.1	monosaccharide transporter	4.32
	Bradi3g56740.2	sucrose transporter 2	1.62
HPHN	Bradi3g46940.1	choline transporter	5.28
	Bradi2g56210.1	magnesium transporter CorA	4.64
	Bradi5g11340.1	chloride channel protein	4.34
	Bradi2g07420.1	L-ornithine transmembrane transporter	3.23
	Bradi1g53780.1	L-tyrosine transporter	-3.46
	Bradi4g17950.1	peptide transporter 2	-3.46
	Bradi2g10800.2	carbohydrate transmembrane transporter	-3.8
	Bradi1g08060.1	calcium channel protein	-3.96
	Bradi3g08690.1	tonoplast monosaccharide transporter 2	-4.69
	Bradi1g76640.2	potassium ion transmembrane transporter	-5.33

Table 4.20. Functional classification of switchgrass transcript IDs related to transport. Significant differentially expressed mycorrhiza-responsive trancript IDs according to their RNA-seq fold changes are shown below. Functional descriptions are based on IDs annotated to MapMan bin 34. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition.

Nutrient	ID	Description	Fold Change
LPLN	Pavir.5KG752300.2	mitochondrial substrate carrier family protein	2.06
	Pavir.1KG122200.1	pleiotropic drug resistance 12	1.99
	Pavir.3KG446700.1	mitochondrial carrier protein	1.59
	Pavir.1NG375300.1	oligopeptide transporter	1.38
	Pavir.1KG186300.3	carbohydrate transmembrane transporter	0.95
	Pavir.1KG239200.1	sulfate transmembrane transporter	0.75
	Pavir.1NG396900.1	amino acid transporter	-1.68
	Pavir.2KG339900.1	integral membrane transporter	-1.84
	Pavir.8KG380700.1	calcium:sodium antiporter	-1.84
	Pavir.6NG126300.2	aminophospholipid ATPase1	-1.84
HPHN	Pavir.8NG286100.1	mannitol transporter	1.81
	Pavir.2NG155700.2	triose-phosphate transmembrane transporter	1.68
	Pavir.5KG492300.1	voltage-gated chloride channel protein	0.77
	Pavir.2KG504400.2	nucleotide-sugar transmembrane transporter	0.53
	Pavir.5KG067400.5	xenobiotic-transporting ATPase	0.53
	Pavir.6KG103900.1	potassium ion transmembrane transporter	0.35
	Pavir.2NG245200.2	cobalt ion transmembrane transporter	0.29
	Pavir.5KG094300.1	mitochondrial substrate carrier	0.28
	Pavir.2KG594100.1	potassium ion transmembrane transporter	-0.25
	Pavir.2KG271800.5	pleiotropic drug resistance 11	-0.82

Table 4.21. Functional classification of prairie cordgrass transcript IDs related to transport. Significant differentially expressed mycorrhiza-responsive contigs according to their RNA-seq fold changes are shown below. Functional descriptions are based on IDs annotated to MapMan bin 34. LPLN: low phosphorus and nitrogen nutrition, HPHN: high phosphorus and nitrogen nutrition. NA descriptions refer to no known function

Nutrient	**ID**	**Description**	**Fold Change**
LPLN	Sobic.007G176100.1	MATE efflux family protein	0.12
	Sobic.006G149400.1	Nucleotide/sugar transporter family protein	-1.19
	Sobic.001G188700.1	OST3/OST6 family protein	-1.98
HPHN	Sobic.006G149400.1	Nucleotide/sugar transporter family protein	4.15

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CHAPTER 5: INTER- AND INTRASPECIFIC FUNGAL DIVERSITY IN THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS

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5.1 Introduction

Plants from practically all environments can form symbiotic relationships with arbuscular mycorrhizal (AM) fungi, all comprised within the phylum, Glomeromycota. AM fungi were previously placed in the Zygomycota, but were later grouped into their own phylum, because molecular data confirmed that this group of fungi is unique and has no obvious affinity to other major phylogenetic groups in the fungal kingdom (Schüßler and Walker 2010). AM associations are formed by approximately 65% of all terrestrial plant species including, but not limited to, a gamut of economically important crops (e.g. corn, rice, soybean, wheat) and even bryophytes including hornworts and liverworts (Pressel et al. 2010; Smith and Smith 2011; Wang and Qiu 2006). The wide distribution of these interactions within the plant kingdom and fossil records suggest that this symbiosis evolved ~ 450 million years ago and played a key role for the evolution of land plants (Taylor et al. 1995).

AM interactions are formed by a large number of different plant species (n > 200,000), but the number of fungal species is relatively low; and has been estimated as less than 350 (Brundrett 2009; Öpik et al. 2013). A high beta diversity among different sampling sites, however, indicates that the global species richness of AM fungi is possibly higher than these estimates (Kivlin et al. 2011). However, the exact number of AM fungal species is difficult to determine, because some species were placed into genera based on older relatively vague descriptions that cannot be verified (Rosendahl 2008).

Plants are also able to form other mycorrhizal associations, such as ectomycorrhizal (ECM), ericoid, or orchid mycorrhizas, with fungi from the phyla Ascomycota and Basidiomycota. However, these relationships are not as prevalent as AM fungal associations and entail a relatively small proportion of the overall mycorrhizal interactions between plants and fungi (Brundrett 2009; Moore et al. 2011). ECM interactions are formed predominantly by woody perennials from cool temperate, boreal, montane to tropical ecosystems (Brundrett 2009). The number of plant species that develop ECM interactions is relatively small with 6,000 – 8,000 species, but ECM fungi exhibit a wide taxonomic range with 20,000 to 25,000 species (Rinaldi et al. 2008; Tedersoo et al. 2010). The ECM fungal diversity can be very high in ecosystems and can consist of hundreds of different ECM fungal species (Buée et al. 2011; Henkel et al. 2012; Newton and Haigh 1998).

Both, the AM and the ECM symbioses play a key role for the nutrient uptake of their host plant, and improve the uptake of P, N, but also of trace elements such as Cu and Zn. In addition, the symbiosis increases the resistance of plants against abiotic (drought, heavy metals, salinity) and biotic (pathogen) stresses (Smith & Read, 2008). But the symbiosis is also costly for the plant, and plants transfer up to 20 % of their assimilated carbon to their fungal partner (Wright et al. 1998). ECM fungi have also saprophytic capabilities, but AM fungi are obligate biotrophs that completely rely on their host plant for their carbon supply and are unable to complete their life cycle without the symbiosis to the host.

AM fungi are not equally beneficial for the host, and mycorrhizal benefits have been described as a mutualism to parasitism continuum (Johnson and Graham 2013;

Johnson et al. 1997; Smith and Smith 2013). However, the mechanisms responsible for these functional differences and the more or less beneficial outcomes for the host are currently unknown. We will discuss here factors that may contribute to the observed high interspecific and intraspecific fungal diversity, and will particularly focus on the AM symbiosis because a better understanding of these processes is critical for a useful application of these fungi in efforts to increase crop production and food security in the future (Rodriguez and Sanders 2015).

5.2 Genetic Diversity of Arbuscular Mycorrhizal Fungi

5.2.1 The arbuscular mycorrhizal fungus – an asexual symbiont?

In the past the standard for 'species' identification of AM fungi was through the determination of morphological traits found amongst resting spore types (Morton and Benny 1990; Mosse and Bowen 1968; Schüßler and Walker 2010). The validity of this methodology is rather limited due to similarities in the spore morphology of different fungal species and significant differences in spore size and color within one fungal species (Bentivenga et al. 1997; Merryweather and Fitter 1998; Morton 1985; Redecker et al. 2013). Another limitation is that the composition of AM fungal communities in colonized roots cannot be accurately identified. Characteristics of fungal structures (arbuscules, vesicles, intercellular hyphae) within colonized roots are not species-specific, and the correlation between the presence of resting spores in the soil and the AM fungal communities in roots is not reliable, because not all identifiable spores may really contribute to the AM community composition of the root. Based on differences in spore morphology, about 250 AM fungal species have been validly described.

Progress in sequencing technologies allow now to classify AM fungal species by morphological data in combination with sequence information of ribosomal RNA genes (SSU or LSU). The availability of these sequence data led to substantial changes in the AM fungal taxonomy and the establishment of several new genera and families within the Glomeromycota (Schüßler and Walker 2010). The new sequencing technologies also revealed that the AM fungal diversity in ecosystems is larger than previously been expected. However, our current understanding of the AM community composition is still limited by the availability of reliable sequence data for all species within the Glomeromycota, and the lack of a universal standard for the identification of operational taxanomic units (OTUs) of AM fungi. When different OTU delineation techniques are compared, one based on the evolutionary origin of monophyletic clades, and the other based on sequence similarities with published sequences, the latter generally leads to a significantly higher number of OTUs and a change in absolute OTU richness (Lekberg et al. 2014). Depending on target gene and sequence similarity cut-off, the number of virtual AM fungal taxa (taxa without morphological analogues) ranges from 300 to 700 in different environmental samples (Kivlin et al. 2011; Öpik et al. 2013).

The biological species concept, however, is difficult to apply to Glomeromycota (Sanders 1999; Sanders 2002). The biological species concept defines species as groups of actually or potentially interbreeding natural populations that occupy a specific niche in nature, and is not solely based on morphological concepts (Mayr 1942, 2000). However, all fungi within the phylum Glomeromycota (in contrast to fungi within the Ascomycota or Basidiomycota) lack any obvious sexual structures and the low morphological diversity within this group of fungi led to the overall assumption that AM fungi are

ancient asexuals. According to evolutionary theory, sexual reproduction is advantageous because the recombination of genes leads to genetic variations and allows the elimination of deleterious mutations and unfavorable traits. The conservation of an asexual lifestyle in AM fungi over such a long co-evolution with plants (~ 450 million years) therefore represents a paradox (Sanders 1999, 2011).

Earlier studies of AM fungi from pot cultures or field collected spores provided no evidence for gene recombination in AM fungi (Rosendahl 2008; Stukenbrock and Rosendahl 2005). But over the past decade the question on whether AM fungi are ancient asexuals without an opportunity for genetic recombination is more controversially discussed. Recent studies revealed that the genomes of several AM fungal species contain genes that are in other organisms involved in sexual reproduction processes. In the transcriptome of *Rhizophagus irregularis* (previously *Glomus intraradices*) (Stockinger et al. 2009) for example, several meiosis-specific genes [*HOP2* (Homologous-pairing protein 2) and *MND1* (Meiotic nuclear division protein 1)] were identified, which are conserved among eukaryotes and are only known to function in eukaryotic meiosis (Tisserant et al. 2012). More than 85% of the core meiotic genes that are involved in the meiosis of *Saccharomyces cerevisiae* can be identified in the AM fungal genome, indicating that AM fungi may be able to undergo a conventional meiosis (Halary et al. 2011).

Recent genomic and transcriptomic surveys also demonstrated the presence of mating type gene homologues and putative sex pheromone-sensing mitogen-activated protein (MAP) kinases in several AM fungal species. In the genomes of *Rhizophagus* spp. and *Glomus cerebriforme* orthologues of the sex pheromone-sensing pathway of *S.*

cerevisiae were identified, which is highly conserved in Asco- and Basidiomycota and involved in the signal transduction pathway between pheromone receptors at the hyphal surface and the transcription factors that regulate mating in these fungi (Halary et al. 2013). However, as long as the exact function of these genes in AM fungi is unknown, their existence is not conclusive evidence for a sort of cryptic sexuality in AM fungi (Corradi and Bonfante 2012). Nevertheless, the identification of these sex-related genes in AM fungi opens up the possibility that the previous view of AM fungi as ancient asexuals and as evolutionary aberration is oversimplified and that cryptic sexuality could be an important pathway in this ecologically important group of fungi (Corradi and Bonfante 2012; Halary et al. 2013).

5.2.2 Arbuscular mycorrhizal fungi have a diverse set of nuclei

AM fungi are unique, because their spores and hyphae are coenocytic and contain multiple nuclei in a common cytoplasm. The number of nuclei in spores can be as high as several hundred or even thousand nuclei per spore, and in the coenocytic mycelium of the fungus up to 100 nuclei can be found per 100 µm of hyphae (Marleau et al. 2011). Genetic diversity in e.g. ribosomal gene sequences of AM fungi can not only be caused by genetic variation among fungal individuals, but also by the heterogeneity found within one individual. It has been hypothesized that in the absence of sexual recombination (see above) evolution should favor individuals with highly divergent genetically different nuclei (Kuhn et al. 2001; Sanders 1999), and indeed individual spores of AM fungi contain a population of genetically divergent nuclei (Hijri et al. 1999; Kuhn et al. 2001; Sanders 1999). It has been hypothesized that AM fungi evolved to be multi-genomic, and that this multi-genomic life style could explain the fitness and the long-term evolutionary

persistence of this group of fungi (Hijri and Sanders 2005; Pawlowska and Taylor 2004). Genetic divergence of spores cannot only be found in ribosomal genes, but also in protein-coding genes, and these genetic variants are passed on from generation to generation through spores (Hijri and Sanders 2005). Kuhn and co-workers assumed (2001) that the genetic diversity is the result of multiple mutations in an otherwise clonal genome, and that recombination events cannot explain the majority of mutations in the genome sequences. Genome polyploidization has also been discussed as a potential origin of the spore divergence in AM fungi (Pawlowska and Taylor 2004), but this view has been questioned by other authors, who reported that even species with a very large nuclear DNA content are haploid (Hijri and Sanders 2005).

5.2.3 The role of hyphal fusions in fungal diversity

Anastomosis, the fusion between encountering AM fungal hyphae could also explain the high nuclei divergence in AM fungi, and there is increasing evidence that these fusion events can contribute to genetic exchange and diversification in AM fungi. Genetically distinct AM fungi can exchange nuclei through anastomosis and it has been demonstrated that genetic markers from each parent are transmitted to the progeny of this hyphal fusion (Croll et al. 2009). However, AM fungi differ in their frequency with which they anastomose, and it has been shown that in *Funnelliformis mosseae* the likelihood that hyphal contacts lead to hyphal fusions is more than 7 times higher than in *F. coronatus* (Pepe et al. 2016). However, even in pairings in which the anastomosis frequency is relatively low, a genetic exchange between the hyphae can be observed (Croll et al. 2009).

Fungal compatibility plays a role in the frequency with which fungal isolates anastomose. While for example a high anastomosis frequency and high compatibility was found between isolates of *Rhizophagus irregularis* that were isolated from a single site (Croll et al. 2009), no anastomosis was observed between geographically distant isolates of *Funneliformis mosseae,* but all these isolates were capable of self-anastomosing (Giovannetti et al. 2003). It has been suggested that similar environments and proximity are important factors for the vegetative compatibility among AM fungi. Successful anastomosis only occurs when the isolates are either genetically similar or from the same habitat (Purin and Morton 2013).

Interestingly it has also been demonstrated that the symbiotic growth phase plays a role for successful anastomosis (Purin and Morton 2013). Before the symbiosis with the host is established and host root and fungus enter the symbiotic growth phase, the fungus undergoes a presymbiotic growth phase that is characterized by spore germination, the exchange of signal molecules between both partners [root exudates (e.g. strigolactones) and so-called "myc-factors" (lipochitooligosaccharides)] (Akiyama and Hayashi 2006; Maillet et al. 2011), and extensive hyphal branching. While in the presymbiotic growth phase anastomosis was relatively unconstraint between hyphae from either genetically identical or different isolates from the same habitat, was hyphal anastomosis suppressed during the symbiotic growth phase (Purin and Morton 2013). This suggest that hyphal anastomosis may fulfill different functions during the presymbiotic or symbiotic growth phase. A potential explanation could be that during the presymbiotic growth phase fungal anastomosis allows to redistribute water and nutrients within the growing hyphal network, while during the symbiotic growth phase anastomosis could cause a significant

slowdown in the water and nutrient transport to the host, and a dilution of the carbon transport from the source (mycorrhizal interface within the root cortical cells) to the sink (growing hyphal tips, and developing spores).

This high genetic diversity among nuclei within one fungal individual may explain the high intraspecific diversity found in AM fungi and the high functional differences and context-dependency of mycorrhizal growth responses. If nuclei with different genetic potential are randomly distributed during spore formation, the offspring of this fungal individual will carry a different composition of nucleotypes compared to the parent or the siblings, and may also differ from the parent or the siblings in its effect on plant growth. Angelard and co-workers (2010) tested this hypothesis and examined the growth response of *Plantago lanceolata* and *Oryza sativa* after inoculation with parental, crossed and offspring lines of the AM fungus *Rhizophagus irregularis* (previously *Glomus intraradices*), and found that the growth of both plants was reduced by an inoculation with crossed lines, compared to the parental lines. Some offspring lines differed also from the other lines in their effect on plant growth. While offspring lines reduced the plant growth of *P. lanceolata* compared to the crossed lines, was the growth of rice significantly increased by the colonization with certain offspring lines. The offspring lines had also a different effect on plant gene expression than the crossed lines (Angelard et al. 2010), and expressed a different fungal phenotype and colonization pattern compared to their respective crossed lines (Angelard and Sanders 2011). This considerable genetic and phenotypic diversity among different single spore lines that share the same parent is also stable over multiple single spore generations (Ehinger et al. 2012).

However, it has also been demonstrated that while there is a high genetic and phenotypic variation among different single spore lines, the differences among subcultured replicates of these single spore lines are small. This suggests that while the genetic potential of each spore is randomly selected during spore development, the phenotypes of these cultures are still relatively stable (Ehinger et al. 2012; Koch et al. 2004). Recently, is was shown that while in crossed isolates the nuclei are inherited by both parents, mitochondria seem to be inherited only by one parent. Based on putative orthologs in the genome of the AM fungus *R. irregularis* to the set of genes involved in the mitochondrial segregation in *S. cerevisiae,* the authors assume that mitochondrial segregation processes are independent from nuclear segregation processes (Daubois et al. 2016).

5.2.4 Is there an effect of endobacteria in fungal diversity?

Recently, it has been demonstrated that endobacteria are widely distributed across the whole phylogenetic range of AM fungi. These mycoplasma-related endobacteria (MRE) are related to the recently discovered bacterial lineage of Mollicutes and live in the fungal cytoplasm. There are indications that this fungal-bacterial symbiosis evolved ~ 400 million years ago (Mondo et al. 2012), and therefore close to the evolution of the AM symbiosis. The bacterial symbiont depends on its host for carbon, phosphate and nitrogen supply, while the dependence of the fungal partner from these endobacteria has been suggested to be relatively low compared to the dependence from the plant partner.

The analysis of the genome of some of these endobacteria revealed typical determinants of symbiotic, pathogenic and free living bacteria that are integrated in an otherwise reduced genome (Ghignone et al. 2012; Naito and Pawlowska 2016). The

endobacterium *Candidatus Glomeribacter gigasporarum* for example is unable to synthesize essential amino acids indicating a strong metabolic dependence of this endobacterium from its fungal partner and an obligate biotrophic life style (Naito et al. 2015). The bacterial genome also contains a substantial proportion of genes that were potentially acquired horizontally from their fungal host. One potential example for a horizontally acquired gene is a SUMO protease that may allow these endobacteria to change the SUMOylation level of fungal proteins (Naito et al. 2015).

The role of MRE in the biology of their fungal hosts is largely unknown (Toomer et al. 2015), but there are indications that the endobacteria have a functioning pathway for the synthesis of folate and of cobalamin (vitamin B12) and contain the genes for a type III secretion system that are used by other pathogenic and symbiotic Gram-negative bacteria to release effector molecules into their host cell (Ghignone et al. 2012). Recent fungal transcriptome and proteome studies demonstrated that endobacteria play an important role during the fungal pre-symbiotic growth phase (Salvioli et al. 2010; Salvioli et al. 2016; Vannini et al. 2016). The endosymbiosis has an influence on fungal growth, calcium signaling and enhances the bioenergetic capacity during the presymbiotic growth phase and plays thereby an important role for the successful establishment of the AM symbiosis with the host plant. Germinating spores that are colonized by endobacteria accumulate proteins involved in DNA replication, transcription and protein synthesis and have higher transcript levels of a Rho-GDP-dissociation inhibitor (Vannini et al. 2016) than control spores. This dissociation inhibitor regulates Rho-GTPases, which are involved in cytoskeletal organization, vesicle trafficking and bud site selection, and are all important processes during fungal growth.

Several genes that are involved in oxidative phosphorylation are upregulated and ATP biosynthesis and fungal respiration are increased in germinating spores with endobacteria, indicating that the colonization with endobacteria increases the bioenergetic potential and the ecological fitness of the fungal host during the critical presymbiotic growth phase (Salvioli et al. 2016; Vannini et al. 2016).

In contrast, the fungal phenotype in the symbiotic growth phase does not seem to be affected by the colonization with the endobacterium *Candidatus Glomeribacter gigasporarum* (Salvioli et al. 2016). However, under consideration that each AM fungal species harbors a distinct group of MRE (Naito et al. 2015) and that there is also a considerable MRE diversity across AM fungal individuals (Agnolucci et al. 2015; Toomer et al. 2015), more research is necessary to evaluate whether bacterial endophytes can also contribute to the functional diversity of AM fungi during the symbiotic growth phase. Based on the currently available evidence it can be assumed that endobacteria at least play a significant role for the successful establishment of the symbiosis, and may have an effect on the AM community composition of the host plant.

In addition to MREs, spores of different AM fungal species and fungal isolates have been shown to be associated with diverse bacterial communities and several of these spore associated bacteria exhibit plant growth promoting capabilities (Agnolucci et al. 2015; Battini et al. 2016). Several bacterial isolates showed for example the capability to produce plant growth hormones and are able to solubilize phosphate from mineral phosphate and phytate. It can be assumed that these bacterial capabilities can also contribute to the mycorrhizal benefits for the host plant, but the composition and effects of these bacterial communities are largely unexplored.

5.3 Host Specificity in the Arbuscular Mycorrhizal Symbiosis

Since the AM fungus is an obligate biotroph that is unable to complete its life cycle without the symbiosis to its host, AM fungal species were seen as generalists with a low host specificity, that will colonize a wide range of host plants (Ehinger et al. 2009). In fact, the low fungus to host species ratio (350 fungal species to 200,000 plant species, see above) has led to the overall assumption that there is a high functional redundancy among fungal species and that the role of inter- and intraspecific fungal diversity does not play an important role for ecosystem functioning (Klironomos 2000).

AM interactions are many-to-many interactions, and each individual host plant is colonized simultaneously with multiple fungal species, and each fungal individual is associated with multiple host plants of the same or of different plant species. These host plants share a common mycorrhizal network (CMN) and it has been demonstrated that AM fungi allocate nutrient resources preferentially to specific host plants within these CMNs (Bücking et al. 2016; Fellbaum et al. 2014; Walder et al. 2012). It has been estimated that in any community between 30 to 50 different AM fungal species could exist (Fitter 2005). For example, in a boreonemoral forest up to 47 fungal taxa were identified (2009; Öpik et al. 2008). If AM fungi are not host-specific all these species could potentially contribute to the AM fungal community composition of a single host plant.

However, new sequencing technologies provide now much more evidence for a host-specificity or at least host-preference of AM fungi. When fungal communities in the roots of forest plant species were compared to the roots of generalist plant species, the fungal taxon richness was significantly higher for forest than for generalist plant species

(28.8 to 13.0 fungal taxa) and the AM fungal community composition differed significantly among these two plant groups (Öpik et al. 2009). Almost half of the fungal virtual taxa that were identified colonized exclusively forest plant species, while only one fungal taxa colonized specifically generalist plant species, and these differences in these fungal communities were unrelated to plant community spatial structure or environmental conditions (Öpik et al. 2009).

Distinct AM fungal communities among different host plant species were also found in a semiarid prairie ecosystem and temperate grasslands (Torrecillas et al. 2012; Valyi et al. 2015). Perennial plant species harbored a lower AM fungal diversity than annual plant species, and half of the AM fungal species that were identified were specific for one plant species (Torrecillas et al. 2012). These data suggest that the host-specificity of AM fungi is higher than previously assumed, and this has also implications for the success and survival of introduced AM fungi and the establishment of designed AM fungal community compositions in agricultural applications for enhanced crop productivity.

5.4 Functional Diversity in the Arbuscular Mycorrhizal Symbiosis

The impact of different AM fungi on plant growth can range from highly mutualistic to antagonistic (Klironomos 2003), and mycorrhizal growth responses have been described as a mutualism to parasitism continuum (Johnson and Graham 2013; Johnson et al. 1997; Smith and Smith 2013). Mycorrhizal growth responses are highly context-dependent, and it has been suggested that particularly the nutrient availability in the soil determines the position of AM fungi along this mutualism to parasitism continuum (Johnson and Graham 2013). High P availabilities in the soil in general reduce

mycorrhizal colonization and mycorrhizal growth benefits for the plant, and negative mycorrhizal growth responses have been discussed as a consequence of the high carbon costs of the symbiosis for the plant that are not counterbalanced by a net gain in phosphate (Peng et al. 1993). However, it has also been suggested that negative mycorrhizal growth responses could be the result of the suppression of the phosphate uptake via the plant pathway (via epidermis and root hairs) which is not compensated for by an increase in the phosphate uptake via the mycorrhizal uptake pathway (via the extraradical mycelium and the mycorrhizal interface) (Smith et al. 2011). AM fungal species differ in the efficiency with which they suppress the plant uptake pathway (Grunwald et al. 2009), and this suppression could lead to an overall reduction in total phosphate uptake and even phosphate deficiency of the plant (Smith et al. 2011). However, a metaanalysis of about 2,000 field and laboratory studies suggest that functional differences not only depend on soil fertility, but also on functional characteristics of the host plants, and the complexity of the soil microbial community, which includes AM fungi and non-mycorrhizal microbial species (Hoeksema et al. 2010). Below we discuss different factors that may contribute to the functional diversity in mycorrhizal growth responses.

5.4.1 Fungal identity

Genetic and functional diversity (see also above) have been observed at all levels of biological organization in AM fungi (Antunes et al. 2011; Hart and Reader 2002; Koch et al. 2006; Munkvold et al. 2004; Powell et al. 2009). However, the reasons for the high functional variability among AM fungi are largely unknown. It has been suggested that fungal growth traits are conserved within one phylogenetic group. For example, Hart and

Reader (2002), who screened different phylogenetic groups within the Glomeromycota for their colonization strategies found that members of the Gigasporaceae tend to extensively colonize the soil, while the colonization of the roots is limited. In contrast, members of the Glomeraceae exhibit a different colonization strategy and extensively colonize the host roots, but show only a relatively low hyphal exploration into the soil. Based on these fungal growth traits, the authors assumed that the phylogenetically determined variability in colonization strategies could also lead to differences in the mechanisms by which these fungi promote host plant growth. The extensive colonization of the root system of the Glomeraceae could suppress the colonization of the root system with root pathogens and thereby contribute to a higher biotic stress resistance of the host, while the better exploration of the soil by hyphae of the Gigasporaceae could have a stronger effect on the nutrient and water uptake of the host. Evidence that fungal growth traits such as levels of root colonization, spore production and extraradical hyphal extension are phylogenetically conserved within the Glomeromycota has also been described by other authors (Antunes et al. 2011; Powell et al. 2009).

Some studies have shown that mycorrhizal growth responses, such as shoot biomass and phosphate and nitrogen contents are positively correlated to fungal growth traits, such as hyphal length, area covered by ERM, hyphal density, or hyphal length per mm of colonized root length (Avio et al. 2006). However, fungal growth traits are not necessarily correlated to mycorrhizal growth benefits or the capability of the AM fungi to increase the phosphate or nitrogen uptake of the plant. In a study, in which the effect of 31 different AM fungal isolates from 10 AM species on plant biomass (*Medicago sativa*) and phosphate and nitrogen uptake was examined, no correlation between fungal growth

traits and mycorrhizal benefits was observed. The authors reported that the capability of AM fungi to increase the growth and nutrient uptake of *Medicago* is not related to the fungal phylogeny, and is relatively widely distributed in the phylum Glomeromycota (Mensah et al. 2015). This is consistent with the results of de Novais et al. (2014), who reported that the ability to promote plant growth is unrelated to the taxonomic classification of AM fungal isolates. This asymmetry between phylogenetically conserved fungal growth traits and evolutionary not conserved host plant effects indicates that other processes such as more efficient nutrient uptake and/or higher nutrient transport rates to the host contribute to the observed functional diversity among AM fungi.

However, there is not only a high interspecific but also intraspecific functional diversity among different isolates of one fungal species (Börstler et al. 2008; Börstler et al. 2010; Koch et al. 2004). Mensah and co-workers (2015), who tested 3 different isolates of 10 fungal species found in all fungal species a high intraspecific variability in the effects on host plant biomass, and phosphate and nitrogen uptake. High within species diversity among different isolates of the same fungal species has been reported in several studies and in symbiosis with different host plant species (Avio et al. 2006; Campagnac and Khasa 2014; de Novais et al. 2014; Koch et al. 2006; Munkvold et al. 2004). The high intraspecific functional diversity can likely be explained by the high genetic variability among different isolates (see also above). In *Funneliformis mosseae* (previously *Glomus mosseae*), for example, a genetic diversity of more than 50% was found among different geographical isolates (Avio et al. 2009). However, similarly high genetic and phenotypic differences can also exist among individuals from one AM fungal population. Five-fold differences in hyphal length were observed among isolates of

Rhizophagus irregularis (previously *Glomus intraradices*) that were isolated from one population (Koch et al. 2004). Hyphal length has previously been used as an important criterion to explain differences in the phosphate uptake by mycorrhizal plants (Jakobsen et al. 1992). The reason for this high functional diversity among different isolates of one AM species is largely unexplored, and should be more strongly considered, when fungal gene expression and function is studied.

5.4.2 Fungal-host compatibility

Before the symbiosis to the host plant can be established, AM fungi undergo a presymbiotic growth phase and respond to their potential host plants with enhanced hyphal branching of germinating spores and a more target-oriented growth of their hyphae (Bücking et al. 2008; Buee et al. 2000). To attract AM fungi, host plants release root exudates that contain several active molecules, e.g. strigolactones, and there are indications that host plants change the composition of their root exudates to attract AM fungi particularly under stressful conditions (Tripathi et al. 2016). Strigolactones for example stimulate hyphal branching, and fungal metabolic activity during the presymbiotic growth phase of the fungus (Akiyama and Hayashi 2006; Akiyama et al. 2005; Besserer et al. 2006; Bücking et al. 2008; Tamasloukht et al. 2003; Tamasloukht et al. 2007). However, there is evidence that the plant genotype plays a critical role in the microbial community composition and that these differences could be the result of quantitative or qualitative changes in the root exudate composition among different plant genotypes (Aira et al. 2010). Consistently, plant genotypes have been shown to differ in their responsiveness to mycorrhizal fungi (Aira et al. 2010; Wang and Bücking 2015).

Branched fungal hyphae on the other hand also secrete a diffusible signal (lipochitooligosaccharides) to the roots, also referred to as `myc-factor` that induces a symbiosis program in the roots, and prepares the roots for colonization. In response to myc-factors specific cells in the roots support the formation of the prepenetration apparatus that provides the fungus with a pathway through the epidermis to the inner cortex where the fungus forms arbuscules (Genre et al. 2005; Parniske 2008). These intracellular highly branched structures are involved in the nutrient to carbon exchange processes between both partners and are characterized by the expression of mycorrhiza-inducible plant phosphate and nitrogen transporters in the periarbuscular membrane, and carbohydrate transporters in the fungal membrane (Breuillin-Sessoms et al. 2015; Gomez et al. 2009; Guether et al. 2009; Helber et al. 2011; Javot et al. 2007). The successful colonization of the root depends on a common symbiosis signaling pathway that is highly evolutionary conserved in mycorrhizal plants. Plants with mutations in this pathway are unable to form a successful symbiosis (Gherbi et al. 2008; Kistner et al. 2005; Parniske 2008). The perception of myc-factors leads to a transcriptional reprogramming of host gene expression (e.g. transcription factors)(Czaja et al. 2012), but whether AM fungi differ in their myc-factor composition and lead to different changes in host plant gene expression is currently unknown.

The mycorrhizal colonization percentage is a common metric to describe the abundance of AM fungal structures in roots, and it is generally assumed that mycorrhizal colonization is positively correlated to host plant benefit. Accordingly, Treseder (2013) found in her meta-analysis an increase in plant biomass and host plant phosphate content with higher mycorrhizal colonization rates. However, differences in the mycorrhizal

colonization are only in part responsible for the variability in host plant responses, and AM fungi differ greatly in the benefit that they provide per root length colonized (Mensah et al. 2015; Treseder 2013).

Mycorrhizal nutrient transport per root length colonized, however, depends on an effective interplay between resource release (carbon from the host plant, and nutrients from the AM fungus) into the mycorrhizal interface and the efficient uptake of resources by both partners from the interface (nutrients by the host plant, and carbon by the fungal partner). If an essential component in these processes is interrupted, a successful symbiosis will not be established. For example, if *MtPt4*, the mycorrhiza-inducible phosphate transporter of *Medicago truncatula* is not expressed, the plant is unable to take up phosphate from the mycorrhizal interface, and arbuscules are prematurely degenerated (Javot et al. 2007). AM fungi can escape this premature degeneration when they are able to transfer nitrogen to their host (Javot et al. 2011). Similarly, if the transcript levels of *MST2*, a high-affinity monosaccharide transporter2 of the AM fungus, are reduced, arbuscules are malformed and the expression of *MtPt4* is reduced (Helber et al. 2011). This indicates that the exchange processes of carbon for nutrients are linked and that both processes are critical for an efficient AM symbiosis. However, AM fungal species differ in their effect on *MtPt4* expression, and there are indications that the expression of this transporter is correlated to the fungal phosphate transport to the host (Fellbaum et al. 2014).

It has been suggested that a reciprocal reward system in which carbon or nutrients are preferentially allocated to more beneficial partners, contributed to the evolutionary stability of the AM symbiosis (Kiers et al. 2011). Plants are able to distinguish between

high-quality and low-quality AM fungi and allocate more carbon to fungi that provide more benefit (Kiers et al. 2011). Similarly, AM fungi transfer more phosphate or nitrogen to plants that are able to provide more carbon benefit (Bücking and Shachar-Hill 2005; Fellbaum et al. 2012; Fellbaum et al. 2014; Hammer et al. 2011). However, resource exchange to multiple partners in the AM symbiosis is not an all-or-nothing process, and fungi still provide nutrients to low quality hosts, and plants still invest carbon into fungal structures of low quality fungal partners (Fellbaum et al. 2014; Kiers et al. 2011). This indicates that resource exchange in the AM symbiosis is controlled by biological market dynamics, and there are indications that the cost to nutrient benefit ratio varies among different host plant species (Walder et al. 2012)

The carbon transport from the host is an important trigger for phosphate and nitrogen transport, and leads to changes in fungal gene expression and in the polyphosphate metabolism of the AM fungus (Bücking and Shachar-Hill 2005; Fellbaum et al. 2012; Hammer et al. 2011; Kiers et al. 2011). Polyphosphates are linear polymers of inorganic phosphate residues linked by phosphoanhydride bonds that play a role for the phosphate and nitrogen transport through the fungal hyphae to the host (Cruz et al. 2007; Kikuchi et al. 2014). It has been suggested that AM fungi control the nutrient release into the mycorrhizal interface by regulating polyphosphate formation and/or remobilization in the intraradical mycelium (Bücking and Shachar-Hill 2005; Ohtomo and Saito 2005; Takanishi et al. 2009). However, the mechanisms that control the resource exchange between partners are only poorly understood, and more research is needed to understand whether and how the processes in the mycorrhizal interface contribute to the functional diversity in the AM symbiosis.

5.4.3 Effects of microbial communities on functional diversity

Our current understanding of functional diversity in the AM symbiosis is primarily based on laboratory experiments and single plant / single fungus interactions. However, plant responses are substantially lower when the plant is colonized with one fungal partner compared with inoculations with multiple fungal species or a whole soil microbial inoculum (with multiple AM fungal species, and non-AM microorganisms) (Hoeksema et al. 2010). The higher plant responses after inoculations with multiple AM species could be the result of (1) a complementarity effect, in which different members of the AM community provide different benefits to the host (Hart and Reader 2002), (2) an establishment of a more beneficial AM fungal community, or (3) a competition effect, in which the competition among fungi for host plant carbon changes the cost to benefit ratio in favor of the host (Bücking et al. 2016). However, there are also reports in which negative effects of multi-fungal communities on host plant growth were observed. Violi et al. (2007) for example demonstrated, that the inoculation with multiple fungi reduced host plant growth and nutrient uptake compared to host plants that were inoculated only with one fungus. Gosling et al. (2016) also reported that an increase in AM fungal diversity does not lead to higher plant growth benefits. This indicates that the general belief that host plant benefits will be higher with more diverse AM fungal communities is not necessarily applicable to all host plants, and that more research is needed to better understand how AM fungal communities (in comparison to single inoculations) affect host plant growth and nutrient uptake.

5.5 Conclusions

There is an increasing interest to apply AM fungi in environmentally sustainable agriculture, but the application of AM fungi is still hindered by the high functional diversity in the AM symbiosis that make host plant responses and/or benefits difficult to predict. Our current understanding of mycorrhizal host plant benefits is mainly based on observations with single AM fungal inoculations that provide only a limited insight into the application potential of specific fungi in certain environments and conditions, and/or for different host plants. In order to identify AM fungi that can provide specific benefits for their host plant, it is critical to better understand the intraspecific genetic diversity within AM fungal species and its effect on host plant benefit. In addition, more research is needed to identify AM fungal communities of specific host plants and under different environmental conditions and to characterize the contributions of individual AM fungi alone and in the community to host plant benefit. For the commercial application of AM fungi or AM fungal communities it is also necessary to examine how specific communities can be established and whether introduced AM fungi are able to survive and to colonize host plants in the presence of an already existing AM fungal community (Rodriguez and Sanders 2015).

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CHAPTER 6: CONCLUSIONS AND FUTURE WORK

Native rhizomatous perennial grasses, such as PCG and SG have a great potential as bioenergy crops. This is possible since they require fewer inputs, produce more energy, and reduce greenhouse gas emissions in comparison to annual cropping systems such as corn and soybean. As mandates for increased biofuel demand increase into the next several decades, the need for maximizing the potential of lignocellulosic crops is vital. While research has been performed into understanding how these plants repond to abiotic conditions, other avenues have been underrespresented. The biotic environment of bioenergy crops can play a crucial role for plant performance and stress tolerance. In the preceding text, we have presented a multi-faceted approach into understanding how plant growth promoting microorganisms can affect the dynamics of bioenergy crops.

We have gained further insight into the microbial community composition of PCG in the Upper Midwest. This multi-faceted biome approach has identified key players in the prokaryotic, eukaryotic, and AMF communities, while corroborating prior AMF community research. We have also shown significant environmental shifts in community diversity, richness, and structure in prokaryotic and AMF microbiomes. While we did perform some analysis into alpha- and beta-diversity, further investigations about how environmental conditions affect community structure should be conducted. This could include collecting more information about the physico-chemical properties of the sampling locations. Additionally, investigating the microbiome at different seasonal times could provide insight into potential temporal variability of microbial communities.

We investigated the impact of AM communities on the biomass production of PCG genotypes found in the Midwest. We found high genotypic variability in the biomass

potential under different nutrient supply conditions and in the mycorrhizal responsiveness of different PCG genotypes. Our finding suggest that benefits of AM symbiosis had strong correlation to an improved phosphate nutrition of the plants, but not nitrogen. To support these findings, additional research should be conducted into the nutritional profile of the remaining substrate.

Using a transcriptomics approach, we examined the impact of the AM fungus, *Rhizophagus irregularis* DAOM197198 on differential expression of mycorrhizal responsive genes in the leaves of PCG, SG, and the model grass species, *Brachypodium distachyon* under two nutrient input conditions. Our results show variations in transcriptomes of each mycorrhizal grass species under low- and high-input nutrent conditions. Changes to carbohydrate metabolism, photosynthesis, sugar transporters, nutrient transporters, and response to disease signalling were most notably observed between these two nutrient conditions. Since we have only observed systemic responses in leaf material, additional DGE research should be conducted in other tissues, including the root system.